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METHODS OF SAMPLING AND TEST FOR GHEE (BUTTERFAT)

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

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Indian Standard

METHODS OF SAMPLING AND TEST FOR GHEE (BUTTERFAT)

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Indian Standard

METHODS OF SAMPLING AND TEST FOR GHEE (BUTTERFAT)

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 16 September 1966, after the draft finalized by the Dairy Industry Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Ghee (milk-fat, butterfat, butter-oil), is the most important dairy product which enters inter-state trade. Due to variation in its composition from region to region and season to season, depending on the type of animal and the food given, the establishment of its purity often involves elaborate analysis, as well as tests for its keeping quality. The present standard describes the methods of sampling and quality control generally needed for such analysis and facilitates the interpretation of results.

0.3 In the formulation of this standard, considerable assistance has been derived from the following publications:

FIL-IDF 6 Acidity of butterfat. International Dairy Federation.

FIL-IDF 7 Refractive index. International Dairy Federation.

FIL-IDF 8 Iodine value. International Dairy Federation.

Directorate of Marketing and Inspection (Ministry of Food and Agriculture). Methods of sampling and testing of butterfat (*ghee*) and butter under agmark. 1953.

B.S. 627 : 1953 Sampling fats and fatty oils. British Standards Institution.

B.S. 684 : 1958 Methods of analysis of oils and fats. British Standards Institution.

Standard Methods of the Oils and Fats—Division of the International Union of Pure and Applied Chemistry. 1964. Butterworths. London.

Official Methods of Analysis of the Association of Official Agricultural Chemists, 9th ed. Washington, 1965, U.S.A.

0.3.1 Full use has also been made of the valuable information received from the National Dairy Research Institute, Karnal.

0.4 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

*Rules for rounding off numerical values (*revised*).

1. SCOPE

1.1 This standard prescribes the methods of sampling, analysis and tests generally used for evaluating the quality of ghee. The specific methods to be used would depend on the object of the analysis.

2. SAMPLING

2.1 Sampling shall be carried out by an experienced person. It cannot be too strongly emphasized that correct sampling is a difficult problem and one which requires the most careful attention to details if the subsequent analysis is to be of value. A sample which is representative of the bulk is essential and is particularly difficult to obtain from a consignment consisting of a large number of packages. However, as a guide to the selection of samples, useful information may be found in documents and certificates normally accompanying the consignments and usually include classification markings. It is recommended that the method given should be adhered to wherever practicable. Particular circumstances may render some modifications of the recommended method necessary.

2.2 Scale of Sampling

2.2.1 *Lot* — All the containers in a single consignment belonging to the same batch of manufacture shall be grouped together to constitute a lot. If a consignment is declared to consist of different batches of manufacture, the batches shall be marked separately and the group of containers in each batch shall constitute separate lots.

2.2.2 The number of containers to be selected for sampling shall depend upon the lot size and shall be in accordance with Table 1.

TABLE 1 NUMBER OF CONTAINERS TO BE SELECTED FOR SAMPLING

Number of Containers in the Lot	Number of Containers to be Selected
N	*n*
1	1
2 to 40	2
41 „ 110	3
111 „ 300	5
301 „ 600	7
601 and above	10

2.2.3 These containers shall be selected at random from the lot. To ensure the randomness of selection, a random number table as agreed to between the purchaser and the supplier, shall be used. In case such a table is not available, the following procedure shall be adopted:

Starting from any container, count them as 1, 2, 3,...up to r and so on, in one order, where r is equal to the integral part of N/n, N being the total number of containers in the lot and n the number of containers to be selected (*see* Table 1). Every rth container thus counted shall be withdrawn to give required number of containers in the sample.

2.2.4 If there is a possibility of wide variation among the different units, for example, in the consignment of ghee from an individual producer, every unit shall be sampled.

2.3 Sample Containers

2.3.1 Wide mouth jar and bottles and tin containers of 50, 100 and 200/250 ml capacities and of following approximate dimensions are convenient to use as sample containers:

Nominal capacity 50 ml	Height 60 × Width 48 × width of the mouth 30 mm
Nominal capacity 100 ml	Height 70 × Width 60 × width of the mouth 44 mm
Nominal capacity 200/250 ml	Height 97 × Width 70 × width of the mouth 60 mm

2.3.2 The jars shall be closed by means of a screw cap lined with butter paper. Bottles shall be glass-stoppered. Tin containers shall be closed with the press-on type of lids. For chemical analysis, bottles may also be closed with rubber stoppers lined with butter paper if organoleptic tests are not to be made.

2.4 Sampling Appliances

2.4.1 The sampling instrument shall be such that it is possible to sample the contents throughout the whole depth of the containers by it.

2.4.2 *Sampling Concentric Tubes* (*Fig. 1A*)—A convenient sampling instrument consists of two concentric tubes closely fitted into each other throughout their entire length, so that one tube may be rotated within the other. A longitudinal opening of about one-third the circumference is cut in both tubes. In one position the tube is open and admits the ghee; by turning the inner tube it becomes a sealed container.

The inner tube may be 19 to 38 mm in diameter and undivided in its length. The two tubes are provided with V-shaped ports at their lower

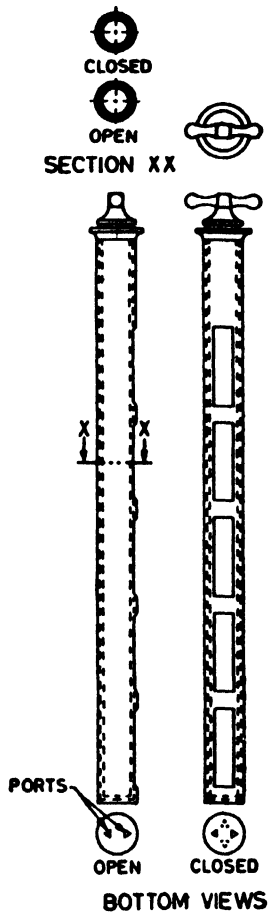


FIG. 1A SAMPLING CONCENTRIC TUBES

ends, so placed that ghee contained in the instrument can be drained through them when the longitudinal openings are open.

The instrument should be inserted closed; it is then opened to admit ghee and finally closed and withdrawn.

2.4.3 *Sampling Plain Tube* (*Fig. 1B*) — The sampling tube may be used when ghee is fluid and is known to be quite uniform. It consists of a metal or thick-walled glass tube which may vary from 20 to 40 mm in diameter and should be 375 to 750 mm long. The upper and lower ends are conical

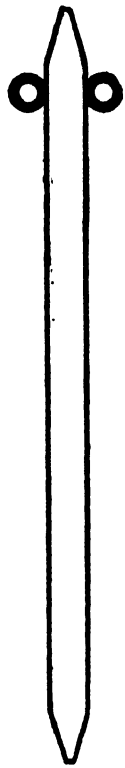


FIG. 1B SAMPLING PLAIN TUBE

and narrowed down to about 6 to 12 mm. At the upper end there are two rings to assist handling.

To take an individual sample the apparatus is first closed at the top with the thumb, or stopper, and lowered until the desired depth is reached; it is then opened for a short time to admit the ghee and finally closed and withdrawn.

2.4.4 The sampling appliances shall be made preferably of stainless steel. The surface of the instruments shall be polished.

2.4.5 All sampling equipment shall be perfectly clean and dry and shall not impart any foreign odour or flavour. Sampling instruments may be cleaned with hot soapy water or other detergent, care being taken to wash away the last traces with scalding hot water. If a source of steam is available the instruments may be given a final cleaning in a jet of steam.

2.5 Sampling Technique

2.5.1 Sampling shall be carried out in such a manner as to protect the sample, the sampling instruments and the containers in which the samples are placed from adventitious contamination, such as rain and dust.

2.5.2 Material adhering to the outside of the sampling instruments shall be removed before the contents are discharged.

2.5.3 A sample shall be drawn from each container to be sampled with the sampling instrument which is inserted through a convenient opening in such a manner as to sample the entire depth of the contents.

2.5.4 All samples from the same consignment shall be put into a clean and dry receptacle, preferably of stainless steel. The contents of the receptacle shall be thoroughly mixed and the required sample drawn into a clean and dry sample container.

2.5.5 The sample container shall be closed, leaving sufficient air space at the top for expansion. On the other hand this space shall not be too large, as air exerts detrimental action.

2.5.6 All samples shall be protected from light and heat, and kept in a cool place.

2.6 Preparation of Composite Sample — Taking equal amount of ghee from each of the containers selected (**2.2.2**), collect at least 300 g of the material as described in **2.5** which shall be mixed and divided into three equal parts. Each part shall be transferred to a separate sample container. One of these composite samples shall be for the purchaser, one for the vendor and the third for the referee. Store the containers at a cool and dark place.

2.7 Transportation and Storage of Samples — Samples should be sent as quickly as possible to the examining laboratory, and should be protected from light and contaminating odour. The sample shall be kept in a cool and dark place.

2.8 Preparation of Sample for Score Card and Analysis

2.8.1 *Sample for Score Card of Ghee* — Testing shall be carried out soon after opening the container. In case of large containers, soon after opening, the contents shall be thoroughly mixed and about 200 g shall be transferred to a glass bottle with a well-fitting stopper. The sample shall not be heated before the score card is prepared.

2.8.2 *Sample for the Determination of Moisture and General Analysis*

2.8.2.1 Mix the sample in the container in which it is received until homogeneous. Carry out this operation in a cool place, away from direct sunlight, and complete it in shortest possible time. In the event of any separation taking place in between, that is, mixing and commencement

of the analysis for moisture, remix the sample. Use this for the determination of moisture.

2.3.2.2 After the determination of moisture place the bottle in a water-bath at a temperature not higher than 50°C till completely melted. Filter through a dried, fluted open-texture 15 cm filter paper (for example, Whatman No. 4) with the help of a hot water funnel, directly into the receiving bottle. Continue the filtration until it is complete, or not more than 3 or 4 ml of ghee remains. The filtered ghee should be bright and clear.

3. QUALITY OF REAGENTS

3.1 Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960*) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the experimental results.

4. DETERMINATION OF MOISTURE CONTENT

4.1 Apparatus

4.1.1 *Moisture Dish* — of aluminium 7 to 8 cm in diameter and 2 to 2·5 cm deep; provided with tight-fitting slip-over cover.

4.1.2 *Desiccator* — containing an efficient desiccant, such as phosphorus pentoxide.

4.1.3 *Air-oven* — preferably electrically heated, with temperature control device.

4.2 **Procedure** — Weigh accurately about 10 g of the sample into a moisture dish which has been dried previously, cooled in the desiccator and then weighed. Place the dish in the air-oven for approximately one hour at 105° ± 1°C. Remove the dish from the oven, cool in the desiccator to room temperature and weigh. Repeat this procedure but keep the dish in the oven only for half an hour each time until the difference between the two successive weighings does not exceed 1 mg. Preserve the dried sample for the determination of insoluble impurities (*see* **10**).

4.3 Calculation

$$\text{Moisture and volatile matter content, percent by weight} = \frac{100\,(W_1 - W_2)}{(W_1 - W)}$$

where

W_1 = weight in g of the dish with ghee before drying,

W_2 = weight in g of the dish with ghee after drying, and

W = weight in g of the empty dish.

*Specification for water, distilled quality (*revised*).

4.4 Accuracy of the Method — The maximum deviation between duplicate determinations shall not exceed 0·1 (percent).

5. DETERMINATION OF COLOUR

5.0 Two methods for measuring colour of ghee are prescribed. The first method using a Tintometer is simple and suitable for routine work. Where more precise information is required the second method using a spectrophotometer shall be used.

5.1 Tintometric Method

5.1.1 *Apparatus*

5.1.1.1 *Tintometer* — preferably with light attachment.

5.1.1.2 *Thermometer* — calibrated from 0° to 50°C.

5.1.1.3 *Tintometer cells* — 0·5-cm and 1-cm.

5.1.1.4 *Water-bath* — maintained at 40° to 50°C.

5.1.2 *Procedure* — Melt the sample as described in **2.8.2.2** and transfer it to the Tintometer cell. Keep the cell in a water-bath and stir the contents with a thermometer. When the sample attains a temperature of 40°C, match the colour against standard glasses in the Tintometer. Express the results as yellow units per cm at 40°C.

5.2 Spectrophotometric Method

5.2.1 *Apparatus*

5.2.1.1 *Spectrophotometer* — A spectrophotometer capable of adjustment to give the following readings on a standard nickel sulphate solution (*see* **5.2.1.3**) at 25° to 30°C, after setting the zero point and after adjusting the 100 percent transmittance point (0 absorbance) against carbon tetrachloride in a cuvette having the outline specified in **5.2.1.2**:

Millimicrons	*Transmittance*
400	Less than 4·0 percent
460	26·2 ± 2·0 percent
510	73·9 ± 1·0 percent
550	54·8 ± 1·0 percent
620	5·2 ± 0·5 percent
670	1·1 ± 0·5 percent
700	Less than 2·0 percent

5.2.1.2 *Matched glass cylindrical cuvettes* — Inside diameter approximately 21·8 mm; outside diameter approximately 24·5 mm. All cuvettes to

be used with a given instrument should be checked with carbon tetrachloride (CCl_4) and the nickel sulphate solution at 550 mμ and give within ±0·6 percent of the same transmittance. The cuvettes should be kept clean and free from scratches.

5.2.1.3 *Standardizing nickel sulphate solution* — Dissolve 200 g of nickel sulphate ($NiSO_4.6H_2O$), analytical reagent grade, in distilled water. Add 10 ml of concentrated hydrochloric acid. Dilute to exactly 1 000 ml in a volumetric flask. The temperature of the solution should be between 25° and 30°C. The nickel content of the solution shall be between 4·40 and 4·46 g of nickel per 100 ml at 25° to 30°C.

5.2.1.4 *Filter paper* — fine porosity, such as Whatman No. 12.

5.2.2 *Reagents*

5.2.2.1 *Carbon tetrachloride* — redistilled if the transmittance differs from distilled water by 0·5 percent at 400 mμ.

5.2.3 *Procedure*

5.2.3.1 *Calibration of the spectrophotometer* — Turn on the spectrophotometer and allow at least 20 minutes' warm-up period before standardizing or making any measurements. After the initial warm-up period, rotate both the control knobs on top of the instrument counter-clockwise to their stop position. Adjust the galvanometer by means of the galvanometer adjustment or by sliding the scale so that an exact zero reading is obtained. Set the wavelength dial to 460mμ. Recheck the zero reading of the instrument, insert a cuvette filled with carbon tetrachloride in the instrument and set the 100 percent transmittance point exactly. Fill a cuvette with the standardizing nickel sulphate solution and read the transmittance of the solution. The reading should fall between 24·2 and 28·2.

In a similar manner set the instrument at 550 mμ and read the transmittance for the nickel sulphate solution. The reading should fall between 53·8 and 55·8.

If the reading at 460 mμ is above 26·2, the reading at 550 mμ should be above 54·8; if the reading at 460 mμ is below 26·2, the reading at 550 mμ should be below 54·8; otherwise adjust the wavelength knob underneath the instrument until both readings are in the same direction above or below the median values established, but within the specific limits.

Set the instrument at 510 mμ and read the transmittance for the nickel sulphate solution. The 510 mμ reading shall be between 72·9 and 74·9

Read the other specified values. All should fall within the limits specified.

5.2.3.2 *Determination* — The sample shall be rendered optically clear and free from water and other suspended impurities. Adjust the temperatue of the sample to 35° to 40°C, fill the cuvette using a sufficient

amount of sample to ensure a full column in the light beam. Place the filled cuvette in the instrument and read the absorbance to the nearest 0·001 at 460, 550, 620 and 670 mμ.

5.2.4 *Calculation*

$$\text{Photometric colour} = 1{\cdot}29\ D_{460} + 69{\cdot}7\ D_{550} + 41{\cdot}2\ D_{620} - 56{\cdot}4\ D_{670}$$

where D is the absorbance.

5.2.4.1 Special instrument scales for reading the four factors involved directly may be used.

6. DETERMINATION OF REFRACTIVE INDEX

6.1 Refractive index is the ratio of the velocity of light in vacuum to the velocity of light in the sample medium; more generally, it is expressed as the ratio between the sine of the angle of incidence to the sine of the angle of refraction when a ray of light of a definite known wavelength (usually 589·3 mμ the mean of the D-lines of sodium) passes from air into ghee. Refractive index of ghee is measured at 40°C to ensure that the sample is completely melted.

6.1.1 Accurate results are obtained by using monochromatic light of a wavelength of 589·3 mμ (the mean of the D-lines of sodium). Diffused white light may be used provided the instrument used is fitted with a suitable compensator. Readings with white light are only accurate when a perfectly colourless and sharp line of demarcation is obtained between the dark and light shades.

6.1.2 The refractive index should be read on an Abbe refractometer which gives the true refractive index or on a butyro-refractometer, which reads on an arbitrary scale at constant temperature as near 40°C as possible.

6.2 Apparatus

6.2.1 *Precision Refractometer* — fitted with an accurate thermometer (reading from 40° to 50°C).

6.2.2 *Hot Water Circulating Device* — to maintain the temperature of the prism constant at 40° ± 1°C.

6.2.3 *Sodium Lamp* — daylight can also be used if the refractometer has an achromatic compensator.

6.3 Reagent — standard fluid for checking the accuracy of the instrument.

6.4 Procedure

6.4.1 The sample shall be rendered optically clear, and free from water and other suspended impurities (*see* **2.8.2.2**).

6.4.2 The correctness of the instrument shall be tested before taking reading by carrying out tests with fluid of known refractive index. At temperature of 40°C, or over, the prisms of most instruments never reach the temperature indicated by the registering thermometer, and at temperatures greatly removed from the standard temperature for the instrument, there is a small error due to the change of the refractive index of the glass. At these high temperatures check the instruments experimentally with a liquid of known temperature coefficient, and apply the correction thus found to instrument readings given by the sample.

6.4.3 It shall be borne in mind that the presence of free fatty acids considerably lowers the refractive index.

6.4.4 Ghee shall completely fill the space between the two prisms, and shall show no air bubbles. The reading shall be taken after ghee has been kept in the prism for 2 to 5 minutes and after it has been ensured that it has attained constant temperature by taking two or more readings.

Take care that the ghee has reached the temperature of the instrument before the reading is taken. Before commencing to take readings circulate through prisms a stream of water at constant temperature and measure accurately the constant temperature at which the readings are taken.

6.4.5 *Use of Abbe Refractometer* — To charge the instrument, open double prism by means of screw head and place a few drops of the sample on prism, or if preferred, open prisms slightly by turning screw head and put a few drops of sample into a funnel-shaped aperture between prisms. Close prisms firmly by tightening the screw head. Allow instrument to stand for few minutes before reading is taken, so that temperature of sample and instrument will be same.

6.4.5.1 Method of measurement is based upon observation of position of border line of total reflection in relation to the faces of a prism of flint glass. Bring this border line into field of vision of telescope by rotating the double prism by means of the alidade in the following manner:

Hold sector firmly and move alidade backward or forward until field of vision is divided into light and dark portion. Line dividing these portions is the 'border line' and, as a rule, will not be a sharp line but a band of colour. The colours are eliminated by rotating screw head of compensator until sharp, colourless line is obtained. Adjust border line so that it falls on point of intersection of cross-hairs. Read refractive index of substance directly on scale of sector. Check correctness of instrument with water at 20°C, the theoretical refractive index of water at 20°C is 1·333 0. Any correction found necessary should be made on all readings. Maximum difference between duplicate determinations shall not exceed 0·000 2 unit of the refractive index.

6.4.6 *Use of Butyro-refractometer* — Place 2 or 3 drops of the sample on surface of lower prism. Close the prism and adjust as in **6.4.5**.

6.4.7 For conversion of refractive index values into butyro-refractometer reading, and *vice versa*, use Table 2.

TABLE 2 BUTYRO-REFRACTOMETER READINGS AND INDICES OF REFRACTION

B. R. Reading	Refractive Index	B. R. Reading	Refractive Index	B. R. Reading	Refractive Index
(1)	(2)	(1)	(2)	(1)	(2)
35·0	1·448 8	40·5	1·452 7	46·0	1·456 5
35·5	1·449 1	41·0	1·453 1	46·5	1·456 9
36·0	1·449 5	41·5	1·453 4	47·0	1·457 2
36·5	1·449 9	42·0	1·453 8	47·5	1·457 6
37·0	1·450 2	42·5	1·454 1	48·0	1·457 9
37·5	1·450 6	43·0	1·454·5	48·5	1·458 3
38·0	1·450 9	43·5	1·454 8	49·0	1·458 6
38·5	1·451 3	44·0	1·455 2	49·5	1·459 0
39·0	1·451 7	44·5	1·455 5	50·0	1·459 3
39·5	1·452 0	45·0	1·455 8		
40·0	1·452 4	45·5	1·456 2		

6.4.8 The refractive index decreases with a rise, and increases with a fall in temperature. If the temperature is not exactly at 40°C, X is added to the observed reading for each degree above or subtracted for each degree below 40°C *pro rata*, where

X for butyro-refractometer = 0·55

X for Abbe refractometer = 0·000 365

Normally the temperature of observation shall not deviate by more than ±2°C.

6.4.9 *Accuracy of the Method* — The maximum difference between duplicate determinations shall not exceed 0·000 2 unit for the refractive index and 0·1 for the butyro-refractometer reading.

7. DETERMINATION OF TITRE

7.1 The titre of ghee represents the highest temperature reached when the liberated water-insoluble fatty acids are crystallized under arbitrarily controlled conditions. The titre is generally taken to represent the

solidification point of the fatty acids, although they actually solidify over a range of temperature.

7.1.1 Ghee is saponified with glycerol-potash solution. The resulting soap is decomposed with sulphuric acid and the liberated water-insoluble fatty acids are separated, washed free from mineral acid and dried. Titre is then determined on these fatty acids.

7.2 Apparatus — The assembly of the apparatus is shown in Fig. 2 and the details of the constituent parts are as follows:

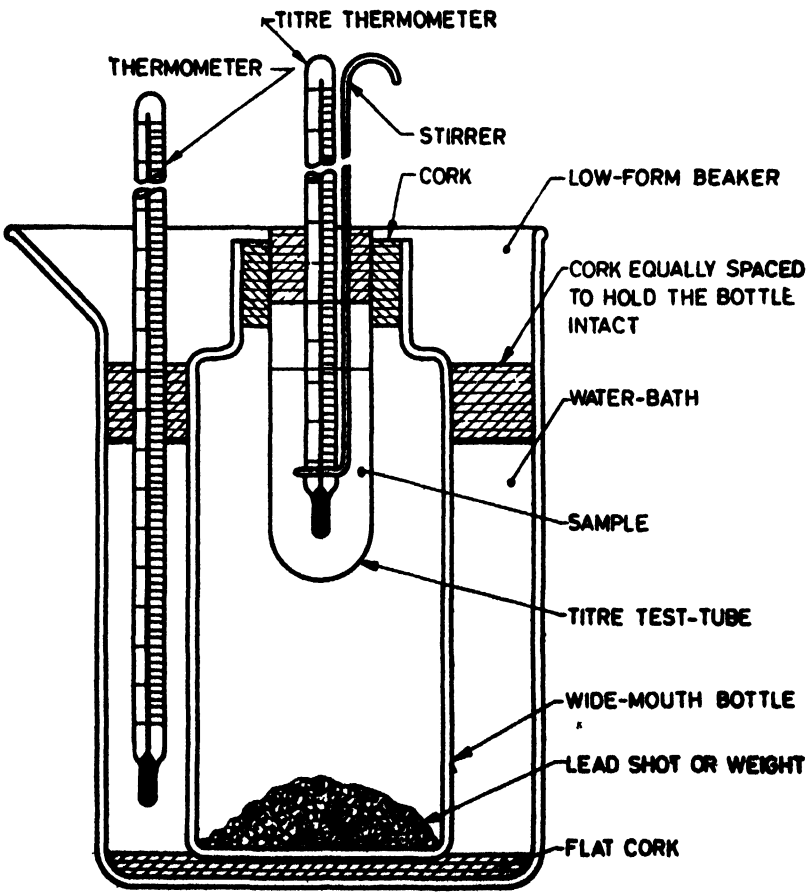


FIG. 2 TITRE ASSEMBLY

7.2.1 *Titre Tube* — of glass, provided at the top with a rim or flange, and having the following dimensions:

External diameter	31·0	to	32·0	mm
Length	90	to	100	mm
Wall thickness	2·0	to	3·0	mm

The tube carries a mark at 57 mm from the bottom to show the height to which the tube is subsequently to be filled, and is inserted in a cork so that it may be held centrally in the wide-mouthed bottle.

7.2.2 *Wide-Mouth Bottle* — of glass, conforming to the following details:

Diameter	60	to	100	mm
Capacity	400	to	800	ml

The bottle shall be loaded with just sufficient lead shots to make it sink and be reasonably stable when it is immersed in the water-bath.

7.2.3 *Water-Bath* — any suitable bath complying with the following requirements:

Water level	10 mm above the 57-mm mark on the titre tube
Temperature of water surrounding the bottle	20° ± 1°C

7.2.4 *Thermometers*

7.2.4.1 *Titre thermometer* — range 0° to 60°C in 0·1 degree steps, calibrated for 45 mm immersion for reading the titre.

7.2.4.2 A general purpose thermometer of suitable range.

7.2.5 *Stirrer* — of glass, stainless steel, or suitable alloy rod of 2 to 3 mm diameter. One end shall be bent in the form of a loop of 19 mm outside diameter at right angles to the shaft of the stirrer. The stirrer is used to agitate the fatty acids immediately before reading the titre.

7.2.6 *Saponification Vessel* — either a flask or beaker of suitable capacity.

7.3 Reagents

7.3.1 *Glycerol-Potash Solution* — Dissolve, with the aid of heat, solid potassium hydroxide in chemically pure glycerine in the proportion 1 : 5 by weight. To avoid foaming, do not heat above 145°C.

7.3.2 *Sulphuric Acid* — approximately 30 percent (*W*/*V*). Add one volume of sulphuric acid (sp gr 1·84) to four volumes of distilled water.

7.3.3 *Methyl Orange Indicator* — 0·05 percent aqueous solution.

7.4 Procedure

7.4.1 *Preparation of Fatty Acids* — Weigh approximately 110 g of glycerol-potash solution into the saponification vessel. Agitate, either by hand or mechanically, and heat to 150°C. Add about 50 ml of sample and reheat to 140° to 150°C. Continue agitation until saponification is complete; this is indicated by the mixture becoming transparent and homogeneous, and is accompanied by a foam which persists for a few minutes when the mixture is allowed to settle.

7.4.1.1 Cool slightly and add 200 to 300 ml of distilled water. Agitate and heat until the soap is completely dissolved. Add carefully, with stirring, 50 ml of dilute sulphuric acid solution. Check for an excess of acid by the addition of a few drops of methyl orange indicator solution; add more acid if necessary. Heat the mixture until the fatty acids separate in the form of a completely melted and clear layer; avoid boiling as the more volatile fatty acids may then be lost.

7.4.1.2 Siphon or draw off the aqueous layer as completely as possible, wash the fatty acids with 500 ml of hot distilled water, allow to settle and again draw off the aqueous layer. Repeat the washings until the wash water is no longer acid to methyl orange. After removal of the last washing, allow the fatty acids to settle for a few minutes and decant through a dry filter paper into a small beaker, taking care to leave behind in the original vessel any water remaining. If visible moisture is still present remove this water by again settling, decanting and filtering the fatty acids. The fatty acids must remain completely melted throughout the filtration.

7.4.1.3 Heat the fatty acids rapidly and momentarily to 130°C to remove any traces of moisture meanwhile continue the stirring. The fatty acids should neither be held at 130°C nor heated to this temperature more than once.

7.4.2 *Determination of Titre* — Fill the titre tube to the 57-mm mark with the fatty acids; when these have cooled to about 15°C above the expected titre, place the tube in the assembly with the flanged rim close to the top of the cork. Insert the titre thermometer to the appropriate immersion mark, the thermometer being supported centrally by a cork, through which also passes the stirrer.

7.4.2.1 Before the temperature of the fatty acids drops to a value of 10°C above its expected titre, commence agitation in a vertical manner at a rate of 100 complete up-and-down motions per minute, the stirrer moving through a vertical distance of about 38 mm. Continue stirring in this manner until the temperature has remained constant for 30 seconds, or has begun to rise within 30 seconds of ceasing to fall. Discontinue stirring immediately and lift the stirrer out of the sample.

7.4.2.2 Observe the rise in temperature; the highest temperature reached after stirring has ceased is the titre.

When making the titre reading, avoid all undue vibration as this will cause the temperature to drop before reaching the maximum.

8. DETERMINATION OF MELTING POINT (SLIP POINT)

8.1 Ghee being a heterogeneous mixture of glycerides, does not have a sharp melting point but for control purposes it is possible to obtain a useful comparison of samples by determining the temperature at which a column of fat of fixed length rises in an open capillary tube. It is essential, however, that the comparison should be carried out under exactly the same conditions and the suitable conditions are described in the procedure.

8.2 Apparatus

8.2.1 *Melting-Point Tubes* — thin-walled, uniformly bored capillary glass tubes open at both ends and with the following dimensions:

Length	50 to 60 mm
Inside diameter	0·8 to 1·1 mm
Outside diameter	1·2 to 1·5 mm

8.2.2 *Thermometer* — with 0·2°C subdivisions and a suitable range. The thermometer should be checked against a standard thermometer which has been calibrated and certified by the National Physical Laboratory, New Delhi, or any other laboratory recognized for such work.

8.2.3 *Melting-Point Apparatus* — beaker with a side-tube heating arrangement. Thiele melting point tube may also be used.

8.2.4 *Heat Source* — gas burner or a spirit lamp.

8.3 Procedure — Melt the sample and filter it through a filter paper to remove any impurities and the last traces of moisture. Make sure that the sample is absolutely dry. Mix the sample thoroughly. Insert a clean melting point tube into the molten sample product so that a column of the material, about 10 mm long, is forced into the tube. Chill the sample in the tube at once by placing the end of the tube containing the sample against a piece of the ice until the fat gets solidified. Place the melting point tube in a test-tube and keep it for one hour either in a refrigerator or in water maintained at 4° to 10°C. Remove the melting point tube and attach with a rubber band or any other suitable means to the thermometer so that the lower end of the melting point tube is even with the bottom of the bulb of the thermometer. Pour water at about 10°C into the beaker or the Thiele tube, and suspend the thermometer in the centre of the apparatus, so that the lower end of the sample column is about 30 mm below the surface of water. Heat the side tube of the apparatus gently, so that the temperature of the water rises slowly at the rate of 2°C per minute till the temperature reaches 25°C, and thereafter at the rate of 0·5°C per

minute. Note the temperature of the water when the sample column commences to rise in the melting point tube. Report the average of two such separate determinations as the melting point, provided that the readings do not differ by more than 0·5°C.

9. SCORE CARD FOR GHEE

9.1 The score card system may be used for judging ghee for competitions exhibitions, etc. A suggested score card for ghee is:

Flavour	50 points
Textura	20 ,,
Colour	10 ,,
Freedom from suspended impurities	15 ,,
Package	5 ,,
Total	100 points

9.1.1 Ghee scoring 91 and above shall be graded as excellent, between 80 and 90 as very good, and between 70 and 79 as good.

9.2 Only pure product can be used for giving score card. The test shall be carried out by a small selected and trained panel of judges. A control sample of type of ghee examined shall be used for comparison.

10. DETERMINATION OF INSOLUBLE IMPURITIES

10.0 Insoluble impurities represent foreign matter exclusive of moisture which are not dissolved by petroleum.

10.1 Apparatus — Conical flask of 250-ml capacity provided with stopper.

10.2 Reagent — Light petroleum of boiling range 40° to 60°C.

10.3 Procedure — Weigh accurately about 20 g of the moisture-free sample (*see* 4.2) into a conical flask. Add 200 ml of light petroleum. Stopper the flask and shake. Allow to stand at room temperature for 30 minutes. Filter the solution through a previously dried and tared 12 cm diameter filter paper.

10.3.1 Wash the filter paper and the flask with light petroleum till fat-free. Remove the filter paper, allow the solvent to evaporate, and dry in an oven at 98° to 100°C for 1 hour. Weigh the filter paper. Repeat drying and weighing until the loss of weight between successive weighings does not exceed 0·000 5 g.

10.4 Calculation

$$\text{Insoluble impurities, percent by weight} = \frac{100\,(W_2 - W_1)}{w}$$

where

W_2 = weight in g of the filter paper and impurities,

W_1 = weight in g of the dry filter paper, and

w = weight in g of the sample taken.

11. DETERMINATION OF ACIDITY

11.1 Apparatus

11.1.1 *Conical flasks* — 250 ml capacity.

11.1.2 *Burette* — with soda lime guard tube.

11.2 Reagents

11.2.1 *Ethyl Alcohol or Rectified Spirit* — 95 percent (*v/v*), sp gr 0·816 0, neutral to phenolphthalein.

11.2.2 *Sodium Hydroxide or Potassium Hydroxide* — 0·1 N aqueous solution accurately standardized against acid potassium phthalate (AR) or oxalic acid (AR).

11.2.3 *Phenolphthalein Indicator* — 1·0 percent solution in 95 percent (*v/v*) ethyl alcohol or rectified spirit.

11.3 Procedure — Weigh 10 g of the sample in a 250-ml conical flask. In a second flask bring 50 ml of alcohol to the boiling point and while still above 70°C neutralize it to phenolphthalein (using 0·5 ml) with 0·1 N sodium hydroxide. Pour the neutralized alcohol on ghee in the flask and mix the contents of the flask. Bring them to boil and while it is still hot, titrate with 0·1 N sodium hydroxide, shaking vigorously during the titration. The end point of the titration is reached when the addition of a single drop produces a slight but definite colour change persisting for at least 15 seconds.

11.4 Acid Value — The number of mg of KOH required to neutralize the free fatty acids present in 1 g of the sample.

$$\text{Acid value} = \frac{5{\cdot}61\,T}{W}$$

where

T = volume of 0·1 N alkali required for titration in ml, and

W = weight in g of sample taken.

11.5 Free Fatty Acids — The acidity of ghee is frequently expressed as the percentage of free fatty acids in the sample, calculated as oleic acid.

$$\text{Free fatty acids} = \frac{2{\cdot}82\ T}{W}$$

11.6 Degree of Acidity — It is the total titratable acidity present in the sample expressed as percentage:

$$\text{Degree of acidity} = \frac{100\ N}{W}$$

where

N = the quantity of alkali used, expressed as ml of 1 N solution.

11.7 Accuracy of the Method — The maximum deviation between duplicate determination shall not exceed 0·2 degree of the acidity or equivalent.

12. DETERMINATION OF SOLUBLE AND INSOLUBLE VOLATILE ACIDS (REICHERT OR REICHERT-MEISSL, POLENSKE AND KIRSCHNER VALUES)

12.1 The method does not determine the total quantities of volatile fatty acids, soluble and insoluble in water, present in combination in fat. The amount of these acids actually determined by the process are dependent on strict adherence to the dimensions of the apparatus and the details of the procedure.

12.2 Definitions

12.2.1 The Reichert-Meissl value (R. M. value) is the number of ml of 0·1 N aqueous alkali solution required to neutralize the water-soluble steam volatile fatty acids distilled from 5 g of ghee under the precise conditions specified in the method.

12.2.2 The Polenske value is the number of ml of 0·1 N aqueous alkali solution required to neutralize the water-insoluble steam volatile fatty acids distilled from 5 g of ghee under the precise conditions specified in the method.

12.2.3 The Kirschner value is the number of ml of 0·1 N aqueous alkali solution required to neutralize the water-soluble steam volatile fatty acids which form water-soluble silver salts distilled from 5 g of ghee under the precise conditions specified in the method.

12.3 Apparatus

12.3.1 *Graduated Cylinders* — 100 ml and 25 ml capacities.

12.3.2 *Pipette* — 50 ml.

12.3.3 The assembly of the apparatus for the distillation is shown in Fig. 3 and 4 and details of the constituent parts are given below:

a) *Flat-bottom boiling flask* (*Polenske*) — The flask shall be made of heat-resistance glass and shall conform to the following details:

Volume contained to bottom of neck	310 ± 10 ml
Length of neck	75 ± 5 mm
Internal diameter of neck	21 ± 1 mm
Overall height	160 ± 5 mm
Diameter of base	45 ± 5 mm

b) *Still-head* — The still-head shall be made of glass tubing of wall thickness 1·25 ± 0·25 mm, and shall conform to the shape shown in Fig. 4, and with the following dimensions:

A	180 ± 5 mm
B	107·5 ± 2·5 mm
C	80 ± 5 mm
D	70 ± 5 mm
E	20 ± 2 mm
F	4 ± 1 mm
G (external diameter of bulb)	37·5 ± 2·5 mm
Internal diameter of tubing	8·0 ± 0·5 mm
Acute angle between slopping part of still-head and vertical	60 ± 2°

A rubber stopper, fitted below the bulb of the longer arm of the still-head, and used for connecting it to the flask shall have its lower surface 10 mm above the centre of the side hole of the still-head.

c) *Condenser* — The condenser shall be made of glass and conform to the following dimensions:

Overall length	520 ± 5 mm
Length of water jacket	300 ± 5 mm
Length of widened part above water jacket	70 ± 10 mm
Wall thickness of widened part	1·25 ± 0·25 mm
Internal diameter of widened part	20 ± 1 mm
External diameter of inner tube within water jacket	12 ± 0·5 mm
Wall thickness of inner tube	1·0 ± 0·2 mm
Wall thickness of outer jacket	1·25 ± 0·25 mm
External diameter of water jacket	30 ± 2 mm

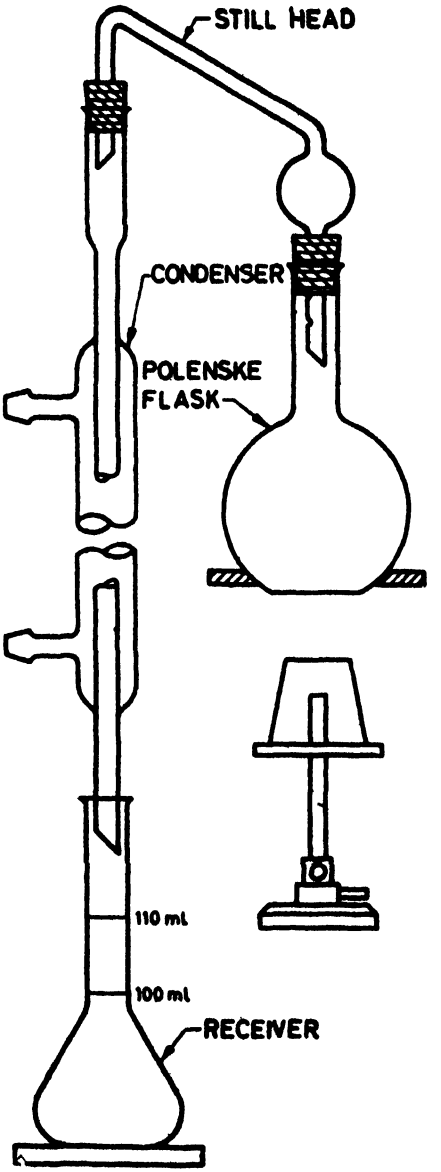


FIG. 3 POLENSKE DISTILLATION APPARATUS

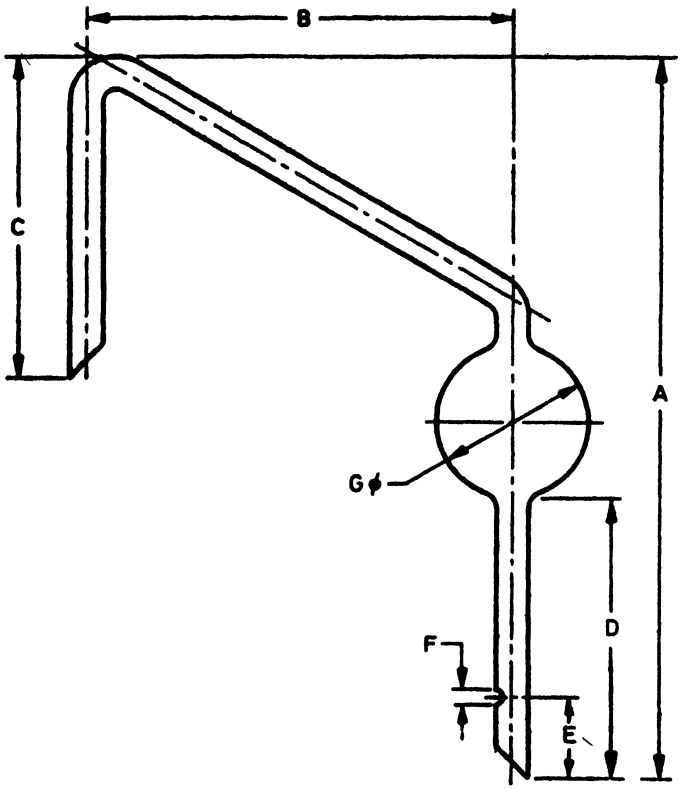


FIG. 4 STILL-HEAD FOR POLENSKE DISTILLATION APPARATUS

d) *Receiver* — The receiver shall be a flask with two graduation marks on the neck, one at 100 ml and the other at 110 ml.

e) *Asbestos-Board* — An asbestos-board of 120 mm diameter and 6 mm in thickness, with a circular hole of about 65 mm in diameter shall be used to support the flask over the burner. During distillation the Polenske flask shall fit snugly into the hole in the board to prevent the flame from impinging on the surface of the flask above the hole. A new asbestos-board may conveniently be prepared by bevelling the edge of the hole, soaking in water, moulding the edge with a flame, and drying.

f) *Gas Burner* — The burner should be sufficiently large to allow the distillation to be completed in the time specified in **12.5.1.3**.

12.3.3.1 The apparatus shall be supported on a retort stand.

12.3.4 *Glass Funnel* — of approximate diameter 6 cm.

12.4 Reagents

12.4.1 *Glycerol* — 98 percent (*w/w*), conforming to AR grade of IS : 1796-1961*.

12.4.2 *Sodium Hydroxide* — 50 percent (*w/w*) solution. Sodium hydroxide is dissolved in an equal weight of water and the solution is stored in a bottle protected from carbon dioxide. The clear portion free from deposit is used.

12.4.3 *Dilute Sulphuric Acid* — approximately 25 ml of concentrated sulphuric acid is diluted to 1 : 1 and adjusted until 40 ml neutralize 2 ml of the 50 percent sodium hydroxide solution.

12.4.4 *Glass Beads* — approximately 1·5 to 2·0 mm in diameter or ground pumice powder, which passes through 250-micron IS Sieve (*see* IS : 460-1962†) and remains on 125-micron IS Sieve (*see* IS : 460-1962†).

12.4.5 *Phenolphthalein Indicator* — 0·5 percent solution is 95 percent (*v/v*) ethyl alcohol, or rectified spirit.

12.4.6 *Ethyl Alcohol* — 95 percent (*v/v*) neutralized to phenolphthalein immediately before use, or neutralized denatured spirit.

12.4.7 *Sodium Hydroxide Solution* — approximately 0·1 N aqueous solution of sodium hydroxide of accurately determined strength.

12.4.8 *Barium Hydroxide Solution* — approximately 0·1 N barium hydroxide solution of accurately determined strength (this solution is needed only if Kirschner value is to be determined).

12.4.9 *Silver Sulphate* — powdered.

12.4.10 *Filter Paper* — Whatman No. 4 (or its equivalent) of 9 cm diameter.

12.5 Procedure

12.5.1 Weigh 5·00 ± 0·01 g of ghee into a Polenske flask. Add 20 g of glycerol and 2 ml of the 50 percent sodium hydroxide solution. Protect the burette containing the latter from carbon dioxide, and wipe its nozzle clean from carbonate deposit before withdrawing solution for the tests; reject the first few drops withdrawn from the burette. Heat the flask over a naked flame, with continuous mixing, until ghee, including any drops adhering to the upper parts of the flask, is saponified, and the liquid becomes perfectly clear; avoid overheating during this saponification. Cover the flask with a watch-glass.

12.5.1.1 Make a blank test without ghee, but using the same quantities of reagents and following the same procedure, again avoiding

*Specification for crude glycerine and refined glycerine.

†Specification for test sieves (*revised*).

overheating during the heating with sodium hydroxide; such overheating would be indicated by darkening of the solution.

12.5.1.2 Measure 93 ml of boiling distilled water, which has been vigorously boiled for 15 minutes, into a 100-ml graduated cylinder. When the soap is sufficiently cool to permit addition of the water without loss, but before the soap has solidified, add the water, draining the cylinder for 5 seconds, and dissolve the soap. If the solution is not clear (indicating incomplete saponification), or is darker than light yellow (indicating overheating), repeat the saponification with a fresh sample of ghee.

12.5.1.3 Add two glass beads, followed by 50 ml of the dilute sulphuric acid, and connect the flask at once with the distilling apparatus. Heat the flask without boiling its contents, until the insoluble acids are completely melted, then increase the flame and distil 110 ml in between 19 and 21 minutes. Keep the water flowing in the condenser at a sufficient speed to maintain the temperature of the issuing distillate between 18° and 21°C.

12.5.1.4 When the distillate reaches the 110-ml mark, remove the flame and replace the 110-ml flask by a cylinder of about 25 ml capacity, to catch drainings. Close 110-ml flask with its stopper, and without mixing the contents, place it in water at 15°C for 10 minutes so as to immerse the 110-ml mark. Remove the flask from the water, dry the outside, and invert the flask carefully, avoiding wetting the stopper with insoluble acids. Mix the distillate by four or five double inversions, without violent shaking. Filter through a dry 9-cm open-texture filter paper (Whatman No. 4) which fits snugly into the funnel. Reject the first runnings and collect 100 ml in a dry volumetric flask; cork the flask and retain the filtrate for titration.

NOTE — The filtrate should be free from insoluble fatty acids. Where liquid insoluble fatty acids pass through the filter, receive the filtrate in a separating funnel, and after separation, draw off the lower (aqueous) layer, leaving behind insoluble acids which have risen to the surface. Add these to the main bulk of the insoluble acids.

12.5.1.5 Detach the still-head and wash the condenser with three successive 15-ml portions of cold distilled water, passing each washing separately through the cylinder, the 110-ml flask, the filter and the funnel, nearly filling the paper each time and draining each washing before filtering the next. Discard the washings. Dissolve the insoluble acids by three similar washings of the condenser, the cylinder, and the filter, with 15 ml of neutralized ethanol, collecting the solution in the 110-ml flask and draining the ethanol after each washing. Cork the flask, and retain the solution for titration, as in 12.5.3.

12.5.2 *Reichert-Meissl, or Soluble Volatile Acid, Value* — Pour 100 ml of the filtrate containing the soluble volatile acids into a titration flask, add 0·1 ml of phenolphthalein indicator and titrate with the barium hydroxide solution until the liquid becomes pink, rinsing the 100-ml flask with the nearly neutralized liquid towards the end of the titration (0·1 N sodium hydroxide solution may be used for the titration if the Kirschner value is not required).

12.5.2.1 If the Kirschner value is to be obtained, the titration flask shall be dried before use; note the actual volume of barium hydroxide solution used; drain the 100-ml flask into the titration flask, close with a cork and continue as in **12.5.4.**

12.5.3 *Polenske, or Insoluble Volatile Acid, Value* — Titrate the alcoholic solution of the insoluble volatile acids after addition of 0·25 ml of phenolphthalein indicator, with the 0·1 N barium or sodium hydroxide solution until the solution becomes pink.

12.5.4 *Kirschner Value* — Add 0·5 g of finely powdered silver sulphate to the neutralized solution reserved in **12.5.2.1.** Allow the flask to stand in the dark for one hour with occasional shaking and filter the contents in the dark through a dry filter. Transfer 100 ml of the filtrate to a dry Polenske flask, add 35 ml of cold distilled water, recently boiled for 15 minutes, 10 ml of the dilute sulphuric acid and 0·1 g of pumice powder or two glass beads. Connect the flask with the standard apparatus and repeat the process as described above, that is, the distillation of 110 ml in 19 to 21 minutes, the mixing (but without the cooling for 10 minutes), and the filtration and the titration of 100 ml of the filtrate with the barium hydroxide solution.

12.6 Calculations

$$\text{Reichert-Meissl value} = 1{\cdot}10\,(T_1 - T_2)$$

$$\text{Polenske value} = T_3 - T_4$$

$$\text{Kirschner value} = \frac{121\,(100 + T_1)\,(T_5 - T_6)}{10\,000}$$

where

T_1 = volume in ml of 0·1 N barium or sodium hydroxide solution used for sample under **12.5.2,**

T_2 = volume in ml of 0·1 N barium or sodium hydroxide solution used for blank under **12.5.2,**

T_3 = volume in ml of 0·1 N barium or sodium hydroxide solution used for sample under **12.5.3,**

T_4 = volume in ml of 0·1 N barium or sodium hydroxide solution used for blank under **12.5.3,**

T_5 = volume in ml of 0·1 N barium hydroxide solution used for sample under **12.5.4,** and

T_6 = volume in ml of 0·1 N barium hydroxide solution used for blank under **12.5.4.**

Polenske values, and to a much slighter extent Reichert values, have been found to be low when determined at low barometric pressures, such

as may occur at high altitudes. The following factors may be applied to values determined at a barometric pressure to convert them to the values determined at normal pressure.

$$\text{Correct Reichert value} = \frac{(\text{Observed value} - .10)\ \log 760}{\log P} + .10$$

$$\text{Corrected Polenske value} = \text{Observed value} \times \frac{760 - 45}{P - 45}$$

where

P = barometric pressure in mm of mercury at the place and time of determination.

12.7 Accuracy of the Method

12.7.1 *Reichert-Meissl Value* — The maximum deviation between duplicate determinations shall not exceed 0·5 units.

12.7.2 *Polenske Value* — The maximum deviations between duplicate determinations shall not exceed 0·3 units.

12.7.3 *Kirschner Value* — The maximum deviations between duplicate determinations shall not exceed 0·5 units.

13. DETERMINATION OF SAPONIFICATION VALUE

13.1 The saponification value denotes the weight of potassium hydroxide, expressed as milligrams, required to saponify completely one gram of ghee. Saponification value is useful in detecting the presence of mineral oils, such as liquid paraffin, in ghee as they are not acted upon by alkali and such a sample does not form a homogeneous solution on saponification.

The saponification value is related to the molecular weight of ghee and from it can be calculated the saponification equivalent, which is the amount of ghee saponified by one gram equivalent of potassium hydroxide, and is equal to 56 100 divided by the saponification value.

13.2 Apparatus — Flat-bottom flask, 200 or 250 ml capacity, resistant to alkali and fitted with a reflux condenser.

13.3 Reagents

13.3.1 *Alcoholic Potassium Hydroxide* — approximately 0·5 N solution in 95 percent (v/v) ethyl alcohol. Dissolve 35 to 40 g of potassium hydroxide pellets in alcohol or if necessary, in a minimum quantity of water (approximately 20 ml) and dilute with ethyl alcohol to one litre. The strength

should be approximate, but not less than 0·5 N. It should be colourless or very pale yellow. Keep in a dark place.

NOTE — Alcoholic solution of potassium hydroxide develops colour due to the presence of aldehydes. Alcohol can be made aldehyde-free by one of the following methods:

a) Saturate alcohol with sodium hydroxide and store for several days, shaking occasionally. Distil.

b) Reflux about 1·2 litres of alcohol with 10 g of potassium hydroxide and 6 g granulated aluminium or aluminium foil, for an hour. Distil and collect one litre, discarding the first 50 ml.

13.3.2 *Phenolphthalein Indicator* — 1·0 percent solution in 95 percent (*v/v*) ethyl alcohol.

13.3.3 *Hydrochloric Acid* — 0·5 N aqueous solution accurately standardized.

13.4 Procedure — Weigh accurately 2·0 ± 0·001 g of ghee into a flask. Add 25 ml, accurately measured, of the alcoholic potassium hydroxide solution. Add 1 or 2 glass beads and boil continuously under a reflux condenser for half an hour to one hour swirling the contents of the flask at frequent intervals. Determine the excess of alkali while the solution is still hot by titration with 0·5 N hydrochloric acid, using 0·5 ml of phenol phthalein indicator.

13.4.1 Make a blank determination upon the same quantity of the potassium hydroxide solution at the same time under the same conditions.

13.5 Calculation

13.5.1 Saponification value $= \frac{28{\cdot}05\ (T_2 - T_1)}{W}$

where

T_2 = volume in ml of 0·5 N acid required for the blank,

T_1 = volume in ml of 0·5 N acid required for the sample, and

W = weight in g of the sample taken.

13.5.2 Saponification Number $= \frac{56\ 100}{\text{Saponification value}}$

14. DETERMINATION OF IODINE VALUE (WIJS' METHOD)

14.1 The iodine value of ghee denotes the percentage by weight of halogen, calculated as iodine, absorbed under the condition of the test.

14.2 Apparatus

14.2.1 *Conical Flask* — 250 ml capacity, preferably with ground-glass stopper, or with good new tight fitting bark corks.

14.2.2 *Burette* — 50 ml, graduated to 0·1 ml.

14.3 Reagents

14.3.0 All reagents shall be of analytical grade.

14.3.1 *Potassium Dichromate* — conforming to IS : 250-1953*.

14.3.2 *Concentrated Hydrochloric Acid* — conforming to IS : 265-1962†.

14.3.3 *Potassium Iodide Solution* — prepare a fresh solution by dissolving 10 g of potassium iodide free from potassium iodate, in 90 ml of water.

14.3.4 *Starch Solution* — Triturate 5 g of starch and 0·01 g of mercuric iodide with 30 ml of cold water and slowly pour it with stirring into one litre of boiling water. Boil for three minutes. Allow to cool and decant off the supernatant clear liquid.

14.3.5 *Standard Sodium Thiosulphate Solution* — approximately 0·1 N. Dissolve approximately 24·8 g of sodium thiosulphate crystals ($Na_2S_2O_3$, $5H_2O$) in water which has been well boiled to free it from carbon dioxide and make up to 1 000 ml. Store the solution in a cool place in a dark-coloured stock bottle with a guard-tube filled with soda lime. After storing the solution for about two weeks, filter, if necessary, and standardize as follows.

14.3.5.1 Weigh accurately about 5·0 g of finely ground potassium dichromate which has been previously dried to a constant weight at 105° ± 2°C into a clean one-litre volumetric flask. Dissolve in water, make up to the mark; shake thoroughly and keep the solution in a cool dark place. For standardization of sodium thiosulphate, pipette 25 ml of this solution into a clean glass-stoppered 250-ml conical flask or bottle. Add 5 ml of concentrated hydrochloric acid and 15 ml of a 10-percent potassium iodide solution. Allow to stand in the dark for 5 minutes and titrate the mixture with the solution of sodium thiosulphate, using starch solution as an internal indicator towards the end. The end point is taken when the blue colour changes to green. Calculate the normality (N) of the sodium thiosulphate solution as follows:

$$\frac{25\ W}{49{\cdot}03\ V}$$

where

W = weight in g of the potassium dichromate, and

V = volume in ml of sodium thiosulphate solution required for the titration.

14.3.6 *Iodine Crystals* — re-sublimed.

14.3.7 *Acetic Acid* — glacial, 99 percent, having a melting point of 16·6°C and free from reducing impurities. Determine the melting point of

*Specification for potassium bichromate, technical and analytical reagent.

†Specification for hydrochloric acid (*revised*).

the acetic acid and test it for reducing impurities as follows:

a) *Determination of melting point* — Take a 15 cm long test-tube and fill it to about two-thirds with the acetic acid. Insert into the acid a thermometer satisfying the requirements specified under **14.4** through a cork stopper fitting the test-tube. The amount of acid should be at least double the quantity required to cover the bulb of the thermometer when the bottom of the latter is 12 mm from the bottom of the test-tube. Suspend this tube within a larger test-tube through a cork. Cool the acid by immersing the assembly in ice water until the temperature is 10°C, then withdraw the assembly from the ice water and stir the acid rather vigorously for a few moments, thus causing the super-cooled liquid to crystallize partially. Take thermometer readings every 15 seconds and consider as the true melting point that temperature at which the reading remains constant for at least 2 minutes.

b) *Test for reducing impurities (potassium permanganate test)* — Dilute 2 ml of the acetic acid with 10 ml of the water and add 2 drops of 0·1 N potassium permanganate solution and maintain at 27° ± 2°C. The test shall be taken as having been satisfied if the pink colour is not discharged at the end of two hours.

14.3.8 *Chlorine Gas* — Dry.

14.3.9 *Iodine Trichloride* (ICl_3)

14.3.10 *Iodine Monochloride* — 98 percent, chemically pure.

14.3.11 *Wijs' Iodine Monochloride Solution* — Prepare this solution by one of the following three methods, and store in a glass-stoppered bottle in a cool place, protected from light:

a) Dissolve 13 g of iodine in one litre of acetic acid, using gentle heat, if necessary, and determine the strength by titration with standard sodium thiosulphate solution. Set aside 50 to 100 ml of the solution and introduce chlorine gas into the remainder until the characteristic colour change occurs and the halogen content is nearly doubled as ascertained again by titration. If the halogen content has been more than doubled, reduce it by adding the requisite quantity of the iodine-acetic acid solution. A slight excess of iodine does no harm, but avoid an excess of chlorine.

Example:

If the titration of 20 ml of original iodine-acetic acid solution requires 22 ml of standard sodium thiosulphate, 20 ml of the finished Wijs' solution should require between 43 and 44 ml (and

not more than 44 ml) of the same sodium thiosulphate solution.

b) Dissolve 8 g of iodine trichloride in approximately 450 ml of acetic acid. Dissolve separately 9 g of iodine in 450 ml of acetic acid using heat, if necessary. Add gradually the iodine solution to the iodine trichloride until the colour has changed to reddish brown. Add 50 ml more of iodine solution and dilute the mixture with acetic acid till 10 ml of the mixture is equivalent to 20 ml of standard thiosulphate solution when the halogen content is estimated by titration in the presence of an excess of potassium iodide and water. Heat the solution to 100°C for 20 minutes, and cool. Prevent access of water vapour in preparing the solution.

c) Dissolve 10 ml of iodine monochloride in about 1 800 ml of glacial acetic acid (chemically pure) and shake vigorously. Pipette 5 ml of this, add 10 ml of potassium iodide solution and titrate with 0·1 N standard sodium thiosulphate solution, using starch solution as indicator. Adjust the volume of the solution till it is approximately 0·2 N.

14.3.12 *Carbon Tetrachloride or Chloroform* — inert to Wijs' solution.

14.4 Procedure — Weigh accurately 0·40 to 0·45 g of the clear ghee in a clean dried conical flask. Dissolve the fat in 15 ml carbon tetrachloride and add by means of a burette exactly 25 ml of the Wijs' reagent. Close the flask with its stopper, mix carefully and leave it standing for one hour in the dark. Add 20 ml potassium iodide solution and approximatly 150 ml of distilled water, and mix. Titrate with 0·1 N sodium thiosulphate solution (use as indicator 2 ml of starch solution), swirling the liquid constantly. Add the starch solution shortly before the end of the titration and shake the contents vigorously. Carry out a blank test, using the same quantities of the reagents.

14.5 Calculation

$$\text{Iodine value} = \frac{12{\cdot}69\ (B - S)N}{W}$$

where

B = volume in ml of standard sodium thiosulphate solution required for the blank,

S = volume in ml of standard sodium thiosulphate solution required for the sample,

N = normality of the standard sodium thiosulphate solution, and

W = weight in g of the material taken for the test.

14.6 Accuracy of the Method — The results of duplicate determinations shall not differ by more than 0·4.

15. DETERMINATION OF UNSAPONIFIABLE MATTER

15.1 The unsaponifiable matter comprises of substances soluble in ghee which after saponification are insoluble in water but soluble in the solvent used for the determination. The important constituents of unsaponifiable matter of ghee are sterols, vitamin A, carotene, and tocopherols.

15.2 Apparatus

15.2.1 *Flat-Bottom Flask* — 250 ml capacity, fitted with a reflux condenser.

15.2.2 *Separating Funnels* — 250 ml cylindrical.

15.2.3 *Glass Funnels* — 5 cm diameter.

15.2.4 *Oven*

15.3 Reagents

15.3.1 *Alcoholic Potassium Hydroxide* — 0·5 N solution in 95 percent (*v/v*) ethanol. The strength should be approximate but not less than 0·5 N, and its colour not darker than pale yellow. Dissolve 35 to 40 g of potassium hydroxide pellets in 20 ml of water and mix the solution with 1 000 ml of 95 percent (*v/v*) ethanol. Allow the solution to stand for several hours, preferably overnight, then decant or filter off the clear supernatant liquid. Keep the filtered solution in a dark place.

15.3.2 *Diethyl Ether* — sp gr at 15·5°/15·5°C, 0·720 to 0·724, residue non-volatile at 80°C not exceeding 0·001 percent.

15.3.3 *Acetone* — residue non-volatile at 80°C not exceeding 0·01.

15.3.4 *Potassium Hydroxide* — approximately 0·5 N aqueous solution.

15.3.5 *Sodium Hydroxide* — 0·1 N solution in ethanol. Dissolve 5 to 6 g of sodium hydroxide pellets in 10 ml of water and mix the solution with 1 000 ml of 95 percent (*v/v*) ethanol. Allow the solution to stand for several hours, preferably overnight, then decant or filter off the clear supernatant liquid. Keep the filtered solution in a dark place.

15.3.6 *Hydrochloric Acid* — sp gr 1·18.

15.3.7 *Ethanol* — 95 percent (*v/v*) sp gr 0·8160.

15.3.8 *Phenolphthalein Indicator* — 1·0 percent solution in 95 percent (*v/v*) ethanol.

15.4 Procedure — Weigh accurately 5 g of the sample to within ±0·01 g in a 250-ml flat-bottom flask, add 50 ml of alcoholic potassium hydroxide solution and two glass beads. Attach the flask to a reflux condenser and

heat on a boiling-water-bath for one hour, swirling at frequent intervals to ensure complete saponification.

15.4.1 Remove the contents of the flask to a 250-ml separating funnel, washing the flask with 50 ml of water. Rinse the flask with 50 ml of diethyl ether and pour the ether cautiously into the funnel. Stopper the funnel, shake vigorously, and allow the funnel to stand until the two layers of liquid separate and clarify. Draw off the aqueous-alcoholic layer into the flask used for the saponification.

15.4.2 Pour the ethereal layer from the top of the funnel into a second 250-ml separating funnel containing 20 ml of water. Extract the aqueous alcoholic soap solution twice more, each time with 50 ml of ether in the same manner, and combine the three extracts in the second funnel.

15.4.3 Wash the ethereal solution twice with 20 ml of water, shaking vigorously on each occasion. Then successively wash with 20 ml of 0·5 N aqueous potassium hydroxide solution, 20 ml of water, 20 ml of 0·5 N aqueous potassium hydroxide solution, 20 ml of water and again with 20 ml of 0·5 N aqueous potassium hydroxide solution, and at least twice more with 20 ml of water. Continue washing with water until the wash water no longer turns pink on addition of phenolphthalein indicator.

15.4.4 Transfer the ethereal solution to a weighed flask and distil to small bulk. Add 2 to 3 ml of acetone and completely remove the solvent from the flask, for example, by means of a gentle current of air, the flask being almost entirely immersed, held obliquely and rotated in a boiling-water-bath. Dry the flask and contents to constant weight at a temperature not exceeding 80°C.

15.4.5 Dissolve the contents in 10 ml of freshly boiled and neutralized 95 percent ethanol and titrate with the 0·1 N alcoholic sodium hydroxide solution, using phenolphthalein indicator.

15.4.6 The titration so obtained should not exceed 0·1 ml. If it does, reject the test and repeat the determination from the beginning.

15.4.7 If there is any reason to suspect the incomplete separation of saponifiable matter, subject the material, as weighed to re-saponification, re-extraction and washing, under the conditions specified in the method. If, on this re-treatment, the amount of unsaponifiable matter obtained is not the same as that weighed in the first determination, within the limits of manipulative error reject the test and repeat the determination from the beginning.

15.5 Calculation

$$\text{Unsaponifiable matter, percent by weight} = \frac{100\ W_1}{W}$$

where

W_1 = weight in g of the residue, and

W = weight in g of the sample taken.

16. DETERMINATION OF VITAMIN A

16.0 The determination of vitamin A may be carried out either by spectrophotometric method or by Carr-Price method. In case of dispute, the spectrophotometric method shall be used.

16.1 Carr-Price Method

16.1.1 *Apparatus*

16.1.1.1 *Photoelectric colorimeter* — instrument with a direct reading deflection type galvanometer, suitable for measuring transmittance or absorbence at 620 mμ.

16.1.2 *Reagents*

16.1.2.0 All reagents shall be of analytical grade.

16.1.2.1 *Vitamin A reference standard* — A solution of crystalline vitamin A of accurately known strength.

16.1.2.2 *Absolute alcohol or isopropanol* — of such spectral purity that when measured in one-centimetre quartz cell against water, it shall show absorbence not greater than 0·01 between 350 and 320 mμ and not greater than 0·05 at 300 mμ.

16.1.2.3 *Potassium hydroxide solution* — 50 percent (*w/v*).

16.1.2.4 *Ether* — peroxide free, redistilled or of anaesthetic grade (*see* IS : 336-1964*). Ether may be maintained free from peroxides by adding wet zinc foil, approximately 80 cm² per litre, cut in strips long enough to reach at least half way up the container, that has been completely immersed in dilute acidified copper sulphate solution for one minute, and subsequently washed with water.

16.1.2.5 *Sodium sulphate* — anhydrous, granular. It shall not absorb vitamin A under conditions of use, and 10 percent solution shall not be acid to methyl red indicator solution.

16.1.2.6 *Antimony trichloride solution* — prepared by dissolving 113·4 g antimony trichloride in 300 to 400 ml of chloroform. Add 5 g of calcium chloride and filter while hot. Dilute the filtrate to 500 ml with chloroform.

16.1.2.7 *Chloroform* — redistilled, discarding the first and the last 10 percent.

16.1.3 *Procedure*

16.1.3.1 *Saponification* — Weigh accurately from the vitamin A reference standard capsules 0·5 to 1·0 g and transfer them to the saponification flask. Reflux for 30 minutes with 40 ml of ethyl alcohol (95 percent *v/v*) and 7 ml of potassium hydroxide solution using all-glass apparatus (rubber stoppers and corks should not be used). Cool, add 30 ml of

*Specification for ether (*revised*).

water and extract three times with 50-ml portions of ether in a separating funnel. Combine the ether extracts in another separating funnel, add 100 ml of water through the ether layer without agitation. When good separation has taken place, after two minutes, remove the aqueous layer. Shake vigorously with 3- to 5-ml portion of water, allow to separate, remove and discard the aqueous layer. If a somewhat resistant emulsion forms, dilute with 100 ml of water to eliminate or decrease this emulsion before discarding the aqueous portion. Wash with two additional portions of 3- to 5-ml of water. Again pour two portions of 100 ml of water through the ether layer and see that the final water wash is not alkaline to phenolphthalein. Evaporate the ether extract on water-bath to about 50 ml. Add 5 to 10 g of sodium sulphate, stir, and allow to settle. Decant into a 50-ml graduated flask, rinse the sodium sulphate with several additional portions of ether, and pour into the flask, diluting to the mark with the final rinse. Test for complete extraction of vitamin A from sodium sulphate by adding a few drops of the antimony trichloride solution to the residue.

16.1.3.2 *Preparation of the calibration curve* — Evaporate a suitable aliquot of the ether solution of the unsaponifiable extract to about 5 ml. Evaporate off the remaining ether at low heat under reduced pressure. Take up the residue in sufficient chloroform to give a concentration having an absorbence of about 0·8 in the photo-electric colorimeter. From this solution make a series of dilutions in chloroform to give absorbance values of 80, 60, 40, and 20 percent of the original absorbance. Determine absorbances of the blue colour formed when one-millilitre aliquot of each of these five solutions plus one millilitre of chloroform is treated with the volume of the antimony trichloride solution, that is, suitable for the operation and hereinafter referred to as 'the fixed volume'. The blank is adjusted to 100 percent transmittance using a tube containing 2 ml of chloroform and the fixed volume of the antimony trichloride solution.

Using a rectangular co-ordinate paper, plot the five absorbances obtained against known quantities of vitamin A and draw up the best smooth curve from the origin through these points. Do not attempt to draw straight line unless the curve is in fact a straight line with the origin at zero. For those instruments that provide other than straight-line curve, check this curve at frequent intervals. For those instruments that do provide straight-line calibration curve, make one reading of the reference solution with each set of sample readings to establish the curve. In the latter case re-establish the calibration curve whenever variation in the reagent or other variables in procedure occurs.

16.1.3.3 *Determination* — Weigh accurately a quantity of the material containing 20 to 45 I.U. of vitamin A (not more than 5 g of the material), then proceed as in **16.1.3.1** and obtain the residue after evaporating the ether under moderate heat and reduced pressure. Dissolve the residue in a definite volume of chloroform so that 2 ml of the chloroform solution with the fixed volume of the antimony trichloride solution would give an

absorbance of about 0·5 to 0·2. Set the instrument at 100 percent transmittance with 2 ml of chloroform and the fixed volume of the antimony trichloride as blank. Place the tube containing 2 ml of the chloroform solution of the residue and add rapidly the fixed volume of the antimony trichloride solution. Record the maximum colorimetric reading. Determine vitamin A from the standard curve and calculate units of vitamin A per 100 g of the sample.

16.2 Spectrophotometric Method

16.2.1 *Apparatus*

16.2.1.1 *Spectrophotometer* — any reliable spectrophotometer with any suitable source of ultra-violet light (incandescent lamp is not a suitable source under 320 mμ). Direct reading spectrophotometer equipped with continuous spectrum source and reading 220 mμ is recommended.

16.2.1.2 *Cells for measuring absorbance in ultra-violet light* — matched quartz cells with one-centimetre internal light path are preferable, but cells of other materials may be used, provided they are sufficiently transparent. If cells are not matched, suitable corrections shall be made.

16.2.2 *Reagents*

16.2.2.0 All reagents stall be of analytical grade.

16.2.2.1 *Absolute alcohol or isopropanol* — of such spectral purity that when measured in one-centimetre quartz cell against water, it shall show absorbance not greater than 0·01 between 350 and 320 mμ and not greater than 0·05 at 300 mμ.

16.2.2.2 *Potassium hydroxide solution* — 50 percent (*w/v*).

16.2.2.3 *Ether* — peroxide-free, redistilled or of anaesthetic grade (*see* IS : 336-1964*). Ether may be maintained free from peroxide by the method given in **16.1.2.4**.

16.2.2.4 *Sodium sulphate* — anhydrous, granular. It shall not absorb vitamin A under conditions of use, and 10 percent solution shall not be acid to methyl red indicator solution.

16.2.2.5 *Chloroform* — redistilled, discarding the first and last 10 percent.

16.2.2.6 *Antimony trichloride solution* — Prepare by dissolving 113·4 g in 300 to 400 ml of chloroform. Add 5 g of calcium chloride and filter while hot. Dilute filtrate to 500 ml with the chloroform.

16.2.3 *Procedure*

16.2.3.1 Weigh accurately a quantity of the material containing 20 to 45 I.U. of vitamin A (not more than 5 g of the material) and transfer it to the saponification flask. Reflux for 30 minutes with 40 ml of ethyl alcohol

*Specification for ether. (*revised*)

(95 percent *v/v*) and 7 ml of potassium hydroxide solution using all-glass apparatus (rubber stoppers and corks should not be used). Cool, add 30 ml of water and extract three times with 50-ml portions of ether in a separating funnel. Combine the ether extracts in another separating funnel, add 100 ml of water through the ether layer without agitation. When good separation has taken place, after two minutes, remove the aqueous layer. Shake vigorously with 3 to 5 ml portion of water, allow to separate, remove and discard the aqueous layer. If a somewhat resistant emulsion forms dilute with 100 ml water to eliminate or decrease this emulsion before discarding the aqueous portion. Wash with two additional portions of 3- to 5-ml of water. Again pour two portions of 100 ml water through the ether layer and see that the final water wash is not alkaline to phenolphthalein. Evaporate the ether extract on water-bath to about 50 ml. Add 5 to 10 g of anhydrous sodium sulphate, stir and allow to settle. Decant into a 50-ml volumetric flask, rinse the sodium sulphate with several additional portions of ether and pour into the flask, diluting to the mark with the final rinse. Test for complete extraction of vitamin A from sodium sulphate by adding a few drops of the antimony trichloride solution to the residue.

16.2.3.2 Evaporate a 10-ml aliquot of ether solution of unsaponifiable extract to about 2 ml. Evaporate the ether using moderate heat and reduced pressure. Take up the residue in sufficient isopropanol or absolute alcohol to give the concentration expected to yield absorbance reading of 0·4 to 0·8 at 325 mμ. Determine absorbance of this solution at 310, 325 and 334 mμ.

16.2.4 *Calculation*

$$\text{Vitamin A content in I.U. per 100 g} = \frac{A\ (\text{corrected})\ 5{\cdot}7}{LW} \times 333$$

where

A (corrected) $= 7\ A_{325} - 2{\cdot}625 \times A_{310} - 4{\cdot}375 \times A_{334}$
(A_{325}, A_{310} and A_{334} represent absorbances at 325, 310 and 334 mμ respectively),

L = length of absorption cell in centimetres, and

W = decimal fraction of unit of sample in one millilitre solution whose absorbance is determined.

17. DETERMINATION OF TOCOPHEROL

17.1 The group of vitamin E consists of the alpha, beta, gamma, and delta-tocopherols. The tocopherols are potent antioxidants.

17.2 Apparatus

17.2.1 *Test-Tube* — 15 × 2·5 cm with reflux condenser.

17.2.2 *Glass Tube* — 12 × 30 mm.

17.2.3 *Photometer with Filter*

17.2.4 *Water-Bath*

17.3 Reagents

17.3.1 *Methyl Alcohol*

17.3.2 *Ethyl Ether* — peroxide-free (ether may be maintained free from peroxide by adding wet zinc foil, approximately 80 cm^2 per litre, cut in strips long enough to reach at least half way up the container, that has been completely immersed in dilute acidified copper sulphate solution for one minute and subsequently washed with water).

17.3.3 *Potassium Hydroxide Solution, Aqueous* — 2 percent.

17.3.4 *Potassium Hydroxide Solution in Methyl Alcohol* — 2 N. Dissolve 112 g of potassium hydroxide pellets in methyl alcohol and dilute to one litre.

17.3.5 *Sodium Sulphate, Anhydrous* — analytical reagent grade.

17.3.6 *α α' Dipyridyl* — 0·5 percent solution in absolute alcohol.

17.3.7 *Hydrochloric Acid* — sp gr 1·16.

17.3.8 *Benzene* — analytical reagent grade.

17.3.9 *Floridin XS Column* — Fill a 12 × 30 mm tube with the purified absorbent. To purify, digest on a boiling water-bath for one hour with hydrochloric acid. Repeat with fresh portions of acid at room temperature. Wash with water until free of acid, then with ethyl alcohol, and with benzene. Dry at room temperature.

17.3.10 *Light Petroleum* — boiling range 40° to 60°C.

17.3.11 *Ethyl Alcohol* — absolute, aldehyde-free (*see* note under **13.3.1**).

17.3.12 *Ferric chloride* ($FeCl_3.6H_2O$) — analytical reagent grade.

17.4 Procedure — Saponify 1 g of the sample in a test-tube attached to a reflux condenser with 2 ml of 2 N methyl alcohol solution of potassium hydroxide for 10 minutes at 72° to 74°C in an atmosphere of nitrogen. Dilute with 8 ml of methyl alcohol, and 10 ml of water, and extract 3 times with 50 ml of peroxide free ether. Wash the combined ether extracts with water, with two percent aqueous potassium hydroxide solution, and again with water until the alkali is removed. Dry the extract which consists of the unsaponifiable matter over anhydrous sodium sulphate and evaporate under vacuum in an atmosphere of carbon dioxide.

17.4.1 *Carotene Removal* — Dissolve the residue in 5 ml of benzene and pass the solution through the Floridin XS column previously wetted with benzene. Wash with benzene until the elute volume is 25 ml. The

absorbent earth is coloured a greenish blue by carotenoids and dark blue by vitamin A.

17.4.2 To 1 ml or a greater volume of the carotene-free unsaponifiable matter solution, add 1 ml of 0·2 percent solution of ferric chloride in absolute ethyl alcohol, prepared fresh from ferric chloride hydrate, and mix. Add 1 ml of a 0·5 percent solution of $\alpha\alpha'$ dipyridyl in absolute ethyl alcohol, mix, and make up to the volume of 25 ml. Prepare a blank in a similar manner. Allow to stand for 10 to 15 minutes and compare the colours in a photometer with a standard solution prepared from pure tocopherol treated with the same amount of reagents. Correct the known and unknown for the blank determination.

18. DETECTION AND DETERMINATION OF DISSOLVED SOAP

18.1 Detection of Soap — Take about 5 ml of liquid ghee in a test-tube and add an equal quantity of hot water. Stopper the test-tube and shake the contents well. Separate the aqueous layer and add a few drops of phenolphthalein solution. A pink colouration indicates the presence of soap.

18.2 Determination of Dissolved Soap

18.2.1 *Apparatus*

18.2.1.1 *Test-tube* — approximately 150 × 40 mm of heat-resistant glass, fitted with ground-glass stopper.

18.2.1.2 *Microburette* — 5 ml capacity.

18.2.2 *Reagents*

18.2.2.1 *Distilled acetone* — containing 2 percent of added water.

18.2.2.2 *Hydrochloric acid* — 0·01 N, accurately standardized.

18.2.2.3 *Bromophenol blue indicator* — one percent solution in 95 percent (*v/v*) ethanol (sp gr 0·816 0).

18.2.3 *Procedure* — Prepare the test solution by adding 0·5 ml of the bromophenol blue indicator to each 100 ml of the aqueous acetone just before use and titrating with 0·01 N acid or alkali until it is just yellow in colour. Weigh 40 g of the sample into the test-tube, which shall have been previously well rinsed with the test solution. Add 1 ml of water, warm on the steam-bath and shake vigorously. Add 50 ml of the neutralized aqueous acetone and after warming on the steam-bath, shake the vessel well and allow the contents to stand until they separate into two layers. If soap is present, the upper layer will be coloured green or blue. Then add 0·01 N acid, preferably from a microburette, until the yellow colour is restored. Continue the process of warming and shaking until the yellow colour of the upper layer remains permanent.

18.2.3.1 It is convenient, but not essential, to run, at the same time a blank on a soap-free sample. Any difference in colour between the upper layers can then readily be perceived.

18.2.4 *Calculation*

$$\text{Dissolved soap, as sodium oleate, percent by weight} = \frac{0{\cdot}304\ T}{W}$$

where

T = volume in ml of 0·01 N acid required, and

W = weight in g of the sample taken.

NOTE — The above method is suitable for the determination of soap in ghee up to 0·05 percent. At higher concentrations it is better to take 4 g ghee and use 0·01 N acid.

19. DETERMINATION OF ANTIOXIDANTS

19.1 Qualitative Tests for Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), Gallates and Nordihydroguaiaretic acid (NDGA)

19.1.1 *Reagents*

19.1.1.1 *Ehrlich reagent* — A 0·5 percent solution of sodium nitrite ($NaNO_2$) in glass distilled water, and a 0·5 percent solution of sulphanilic acid in glass distilled water containing 50 percent concentrated hydrochloric acid, are kept refrigerated. The nitrite solution should be freshly prepared every 3 weeks. The 2 solutions are mixed each working day in a ratio of 1 : 100 of nitrite to sulphanilic acid to perform the reagent (diazobenzenesulphonic acid).

19.1.1.2 *Ethyl alcohol* — 72 percent (*v/v*).

19.1.1.3 *Sodium hydroxide* — 1 N.

19.1.2 *Procedure*

19.1.2.1 One ml of melted fat is shaken in a test-tube with 2 ml of 72 percent ethyl alcohol. The emulsion formed is shaken with 1 ml of Ehrlich reagent, then immediately with 1 ml of normal sodium hydroxide solution:

a) *BHA* — The development of a red-purple colour indicates the presence of BHA and shows an absorption maximum at 535 mμ.

b) *BHT* — Presence of BHT gives a distinct salmon-pink colour with an absorption maximum near 505 mμ. The colour produced with BHT develops very slowly under the conditions mentioned for BHA.

c) *Propyl gallate* — Under the conditions of the test, propyl gallate is found to give a yellow colour, which fades rapidly and gives no

measurable maximum in the visible range. Other gallates behave similarly.

d) *NDGA* — NDGA yields a wine red colour, which rapidly changes to brown and brown yellow.

19.2 Estimation of Antioxidants

19.2.1 Methods are prescribed for determining the antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), nordihydroguaiaretic acid (NDGA), and all combinations except those containing both propyl gallate and nordihydroguaiaretic acid. Butylated hydroxyanisole and butylated hydroxytoluene are separated from the fat and the other antioxidants by distillation with superheated steam. The distillate is analyzed for the sum of butylated hydroxyanisole and butylated hydroxytoluene with ferric chloride-2, 2'-bipyridine and for butylated hydroxyanisole with 2, 6-dichlorequinonechloroimide, thereby permitting butylated hydroxytoluene to be determined by difference. Nordihydroguaiaretic acid and propyl gallate are extracted from a carbon tetrachloride solution of the fat using 50 percent ethyl alcohol and are determined with ferrous sulphate buffered to an appropriate *p*H. Butylated hydroxyanisole and butylated hydroxytoluene, although partially extracted with 50 percent ethyl alcohol, do not react with ferrous sulphate.

19.2.1.1 In a separate outline propyl, octyl and dodecyl gallate are determined absorptiometrically in a sodium acetate buffer solution with ferrous tartrate, which is specific for the gallates. After solution of the fat in light petroleum, propyl gallate is extracted with water and the higher gallates with absolute methanol. With the extraction methods described 95 to 97 percent recovery of antioxidant was possible.

19.2.2 *Apparatus*

19.2.2.1 *Distillation apparatus for BHA and BHT* — The distillation apparatus consists of a steam generator consisting of a 1 000-ml Erlenmeyer flask containing water and several glass beads. The rubber stopper in the flask has an outlet tube ending in a 12/5 socket joint. Water is boiled on an electric heater. The superheater consists of a glass coil placed in a 1 000-ml beaker half full of wax (mp60°C, smoke point 27°C, flash point 36°C), and provided with a thermometer. The distilling flask is made from a 50/50 standard-taper joint, a 12/5 and a 28/15 ball joint. The distilling flask is heated in a 1 000-ml beaker containing approximately 800 ml of bath wax. During a distillation the inlet and outlet tubes of the superheater and distilling flask are wrapped in glass wool. The condenser is 61 centimetre long with a 28/15 socket joint. The distillate is filtered and collected in a 250-ml glass-stoppered graduate.

19.2.2.2 *Photoelectric colorimeter* — with 515, 530, 550 and 620 mμ filters, and cells.

19.2.2.3 *Separating funnels* — 150 ml and 250 ml capacities.

19.2.3 *Reagents*

19.2.3.0 All reagents should be of analytical grade.

19.2.3.1 *Carbon tetrachloride*

19.2.3.2 *Ethyl alcohol* — Add approximately 0·1 percent potassium hydroxide and potassium permanganate to commercial absolute alcohol and distil in all-glass apparatus. The distillate is 100 percent alcohol and is diluted volume for volume to obtain the 50 and 25 percent alcohol.

19.2.3.3 *Barium hydroxide* — Prepare one percent barium hydroxide [$Ba(OH)_2$, H_2O] in boiled distilled water. This reagent shall be kept in a tightly stoppered bottle.

19.2.3.4 *Ammonium hydroxide* — concentrated.

19.2.3.5 *Ferrous sulphate* — 0·04 percent ferrous sulphate ($FeSO_4$. $7H_2O$) in distilled water, freshly prepared.

19.2.3.6 *Sodium carbonate-bicarbonate buffer* — Prepare 5·3 percent anhydrous sodium carbonate and 4·2 percent sodium bicarbonate in distilled water.

19.2.3.7 *Ammonium acetate* — Prepare a solution containing 2 percent of ammonium acetate in distilled water.

19.2.3.8 *Calcium chloride* — 20 mesh.

19.2.3.9 *Ferric chloride* — Prepare fresh 0·2 percent of ferric chloride ($FeCl_3$. $6H_2O$) in distilled water.

19.2.3.10 *2,2'-Bipyridine* — Dissolve 200 mg of 2,2'-bipyridine in 0·5 ml of 100 percent ethyl alcohol and dilute to 100 ml with distilled water. If the 2,2'-bipyridine is brownish, sticky, or possesses a strong odour, it should be purified as follows:

Dissolve 10 g of 2,2'-bipyridine in 10 ml of warm, purified ethyl alcohol and add approximately 250 ml of cold distilled water. Allow to stand in a refrigerator overnight. Filter the flake like bipyridine crystals and wash with cold distilled water. The purified 2,2'-bipyridine should be white flakes with a faint sweet odour. Retain the supernatant in a refrigerator where a second crop of crystals is obtained upon evaporation of the water. Purify these crystals as above.

19.2.3.11 *Borax buffer* — Prepare 2 percent borax ($Na_2B_4O_7$. $10H_2O$) in distilled water.

19.2.3.12 *n-Butyl alcohol*

19.2.3.13 *Methanol* — Boil one litre of absolute methanol under reflux for 1 hour with 8 g of solid potassium hydroxide and 5 g of aluminium powder and then distil.

19.2.3.14 *Ferrous tartrate solution* — Dissolve 100 mg of ferrous sulphate and 500 mg of analytical reagent grade potassium sodium tartrate (Rochelle salt) in 100 ml of distilled water. This reagent should be freshly prepared for each series of determinations.

19.2.3.15 *Sodium acetate solution* — One percent. Dissolve 10 g of sodium acetate ($CH_3COONa.3H_2O$) in one litre of distilled water.

19.2.3.16 *Isoamyl alcohol*

19.2.3.17 *Light petroleum, boiling range 40° to 60°C* — Shake one litre of the light petroleum with small amounts of concentrated sulphuric acid until colourless and then wash it several times with one percent sodium hydroxide solution and finally with distilled water until it is free from acid. Dry the solvent with anhydrous sodium sulphate, filter and distil.

19.2.4 *Procedure*

19.2.4.1 *Analysis of propyl gallate and nordihydroguaiaretic acid* — Weight 20 g of the ghee into a 250 ml separatory funnel and dissolve in 40 ml of carbon tetrachloride. Gentle warming may be necessary to complete the solution. Extract this solution with 70 ml of 50 percent ethyl alcohol by repeatedly inverting the funnel for 5 minutes, at the rate of approximately 120 inversions per minute. Run the entire contents of the separating funnel into a 250 ml centrifuge bottle and centrifuge for 10 minutes at 1 500 rev/min. Pour the upper alcoholic layer into a beaker. A portion of this 50 percent alcoholic extract is used for the qualitative analysis and the remainder is then diluted to 25 percent alcohol for the quantitative analysis:

a) *Qualitative analysis*

1) *Nordihydroguaiaretic acid* — Pipette 10 ml of the 50 percent alcoholic extract into a test-tube (15 × 1·5 cm). Add 1 ml of barium hydroxide reagent, shake immediately, and look down the length of the tube against a white background. If there is more than 0·001 percent of nordihydroguaiaretic acid in the fat, a blue colour will form and fade rapidly. If there is more than 0·002 percent of propyl gallate in the fat, a transitory green colour forms. If both propyl gallate and nordihydroguaiaretic acid are present in the same sample, a green colour forms first and quickly fades; then the blue nordihydroguaiaretic acid colour forms and fades. To obtain a positive test in the presence of propyl gallate, at least 0·003 percent of nordihydroguaiaretic acid should be present in the fat.

2) *Propyl gallate* — Pipette another 10 ml of the 50 percent alcohol extract into a test-tube and add 1 ml of concentrated ammonium hydroxide. If 0·000 1 percent of propyl gallate is present in the fat a pink to red colour forms. This colour is

stable for 3 to 5 minutes depending upon the propyl gallate concentration.

b) *Quantitative analysis* — Pipette 25 ml of the 50 percent alcoholic extract into a 50 ml centrifuge tube. Add 25 ml of water, mix and centrifuge at 2 000 rev/min for 15 minutes, or until a clear solution is obtained. Pour off the clear 25 percent alcoholic solution into a beaker and use this solution for the quantitative analysis.

1) *Nordihydroguaiaretic acid* — Pipette three different aliquots of the diluted alcoholic extract (25 percent ethyl alcohol) into 18-mm colorimeter tubes and make up to 12 ml with 25 percent ethyl alcohol. Add 1 ml of ferrous sulphate reagent and 1 ml of sodium carbonate-bicarbonate buffer. Measure the absorbency after 10 minutes with a photo-electric colorimeter using a 515 mμ filter. All absorbencies should be measured relative to a reagent blank.

Prepare a reference curve over a range of 50 to 500 μg of nordihydroguaiaretic acid by replacing the alcoholic extract in the above procedure with aliquots of a standard nordihydroguaiaretic acid solution in 25 percent ethyl alcohol. Under these conditions, the observed absorbency divided by a *k*-value of 0·001 41 gives the concentration of nordihydroguaiaretic acid in micrograms per aliquot used.

2) *Propyl gallate* — Pipette three different aliquots of the diluted alcoholic extract (25 percent ethyl alcohol) into 18-mm colorimeter tubes and make up to 12 ml with 25 percent ethyl alcohol. Add 1 ml of ferrous sulphate reagent and 1 ml of ammonium acetate buffer, and mix. Measure the absorbency after 10 minutes with a photo-electric colorimeter using a 515 mμ filter. All absorbencies should be measured relative to a water blank. Prepare a reference curve over the range of 30 to 300 μg of propyl gallate per aliquot. Using this procedure, the observed absorbency divided by a *k*-value of 0·002 05 gives the concentration of propyl gallate in micrograms per aliquot used.

19.2.4.2 *Analysis of butylated hydroxyanisole and butylated hydroxytoluene*

a) *Distillation* — Place 16 grams of anhydrous calcium chloride (reagent grade) and 10 ml of distilled water in the distilling flask; cool to approximately room temperature and weigh 5 grams of the ghee sample in the flask. Lightly grease the ground-glass joint and place the top on the distilling flask.

Before starting the distillation, heat the bath for the distilling flask to 160° ± 10°C, and the superheater bath 200° ± 20°C and

adjust the steam generator to distil approximately 4 ml of water per minute. Maintain these conditions throughout the entire distillation.

As soon as these conditions are fulfilled, connect the superheater and the condenser to the distilling flask. Start the distillation by connecting the steam generator to the superheater and immediately place the bath around the distilling flask. Collect the distillate in a 250-ml glass-stoppered graduate, filtering the distillate through a 9-cm Whatman filter paper No. 54 or equivalent as it collects. The rate of distillation should be such that 125 ml of distillate collects in 30 ± 5 minutes.

When 125 ml of distillate has been collected, stop the distillation by disconnecting the distilling flask from the superheater and removing the bath around the distilling flask. When the mouth of the condenser has cooled, disconnect it from the distilling flask and drain the water from the water jacket. Wash the condenser and filter paper thoroughly, using six 10-ml portions of hot (60° ± 5°C) 100 percent ethyl alcohol, allowing the alcoholic washings to filter into the distillate. Continue washing the filter with hot alcohol until the combined volume of distillate and washings is 250 ml when cooled to room temperature.

b) *Analysis of distillate*

1) *Butylated hydroxyanisole (2, 6-dichloroquinonechlorimide method)*— Pipette three different aliquots of the distillate (50 percent alcohol) into 18-mm colorimeter tubes and make up to 12 ml with 50 percent ethyl alcohol. Add 2 ml of the 2, 6-dichloroquinonechlorimide reagent and 2 ml of borax buffer and mix. After 15 minutes, add 5 ml of *n*-butyl alcohol to each tube, mix, and measure the absorbency with a photo-electric colorimeter using a 620 mμ filter. Measure all absorbencies relative to a reagent blank.

Prepare a reference curve over a range of 10 to 50 μg of butylated hydroxyanisole. The concentration of butylated hydroxyanisole, in microgram per aliquot used, is obtained by dividing the observed absorbency by a *k*-value of 0·010 2.

2) *Butylated hydroxyanisole plus butylated hydroxytoluene (ferric chloride-2, 2'-bipyridine method)* — All solutions shall be cooled to room temperature before starting this analysis. Pipette duplicate aliquots of the alcoholic distillate (50 percent ethyl alcohol) into 50-ml glass-stoppered Erlenmeyer flasks rendered impervious to light with black tape, and make up to 8 ml with 50 percent ethyl alcohol. Add 2 ml of ferric chloride reagent and 2 ml of 2, 2'-bipyridine reagent to each flask and mix. Thirty minutes after the addition of the

ferric chloride reagent add 5 ml of *n*-butyl alcohol and mix. Thirty-five minutes after the addition of the ferric chloride reagent, pour the contents of the flask into an 18-mm colorimeter tube and, after a further 2 minutes, measure the absorbency in a photo-electric colorimeter using a 515 mμ filter. All measurements are made relative to a reagent blank. The absorbency is a measure of the sum of butylated hydroxyanisole and butylated hydroxytoluene. Prepare a reference curve for butylated hydroxytoluene over a range of 10 to 50 μg with each set of analysis. This is necessary since the *k*-value for butylated hydroxytoluene varies with temperature; *k*-values were found to range from 0·010 8 at 22°C to 0·014 2 at 30°C.

Prepare a reference curve for butylated hydroxyanisole over a range of 10 to 50 μg. Under the above conditions a *k*-value of 0·011 4 was obtained. Divide the absorbency obtained with the 2, 6-dichloroquinonechlorimide reagent by the aliquot volume and by the 2, 6-dichloroquinonechlorimide *k*-value for butylated hydroxyanisole to obtain the concentration of butylated hydroxyanisole in micrograms per millilitre of distillate. Multiply this value by the ferric chloride-2, 2'-bipyridine *k*-value for butylated hydroxyanisole and by the number of millilitres of distillate used in the ferric chloride-2, 2'-bipyridine reaction. This figure represents the absorbency due to butylated hydroxyanisole in the ferric chloride-2, 2'-bipyridine reaction. Subtract this latter figure from the measured absorbency in the ferric chloride-2, 2'-bipyridine reaction to find the absorbency due to butylated hydroxytoluene. Calculate the amount of butylated hydroxytoluene in the distillate.

Example:

Weight of fat sample	= 5·0 g	
Total volume, distillate + washings	= 250 ml	
2, 6-Dichloroquinonechlorimide reaction *K*BHA	= 0·102	
Aliquot volume	= 12 ml	
Absorbency	= 0·260	
Ferric chloride-2, 2'-bipyridine reaction *K*BHA	= 0·011 4 = 8 ml	aliquot volume
*K*BHT	= 0·0122 = 0·398	absorbency

Calculation:

Concentration of BHA per ml of the distillate $= \frac{0{\cdot}260}{0{\cdot}010\,2 \times 12} = 2{\cdot}27\ \mu g$

Absorbency per aliquot due to BHA in $FeCl_3$-2, 2'-bipyridine reaction $= 2{\cdot}27 \times 0{\cdot}114 \times 8 = 0{\cdot}207$

Absorbency per aliquot due to BHT in $FeCl_3$-2, 2'-bipyridine reaction $= 0{\cdot}398 - 0{\cdot}207 = 0{\cdot}191$

Micrograms of BHT in the distillate $= \frac{0{\cdot}191}{0{\cdot}012\,2} \times \frac{250}{8} = 489$

Concentration of BHT in the ghee sample $= \frac{489}{5 \times 10^6} \times 100 = 0{\cdot}009\,8$ percent

Concentration of BHA in the ghee sample $= \frac{2{\cdot}27 \times 250}{5 \times 10^6} \times 100$

$= 0{\cdot}011\,3$ percent

NOTE—The presence of BHA, BHT, tocopherol in ghee causes no error in the analysis of propyl gallate and NDGA, or *vice versa*.

19.2.4.3 *Determination of propyl, octyl and dodecyl gallates*

a) *Extraction* — Dissolve 50 g of ghee in light petroleum. Extract the solution 5 times successively with 20-ml portions of distilled water at 30°C, taking 2 minutes over each extraction. Separate the phases and filter the aqueous layer into a 110-ml calibrated flask; then wash the filter with water and add the washing to the filtrate in the flask until the mark is reached.

Shake the fat-light petroleum layer, from which the water has, as far as possible, been separated and removed, first with 55-ml portion and then with four 15-ml portions of methanol, taking 2 minutes over each extraction. With some samples partial crystallization of the glycerides may occur owing to cooling. If so, the extraction should be carried out at 25°C, for example, by gentle-heating of the separating funnel. It is essential to wait at least 5 minutes after each extraction in order to obtain good separation of the layers, which may be assisted by swirling the separating funnel. At least 30 minutes shall be allowed after the last extraction before running off the lower layer.

Transfer each extract as completely as possible to a 150-ml separating funnel. Add 3 ml of distilled water to the combined extracts and shake well. After 30 minutes have elapsed add the final residue of methanol that has separated from the fat-light petroleum layer and shake the separating funnel again. Run off the clear lower layer into a 110-ml calibrated flask, add sufficient methanol to make up to the mark. When necessary, a 125-ml calibrated flask may be used instead.

b) *Analysis of the extract*

1) *Estimation of propyl gallate* — Pipette 10 ml of the water extract in a 50 ml calibrated flask. Add 1 ml of ferrous tartrate solution, fill up to the mark with sodium acetate solution and mix well. After 10 minutes measure the optical density of the solution at 530 mμ relative to that of water, using an absorptiometer and a 2-cm cell. To determine the optical density of the blank, dilute 10 ml of water extract, without ferrous tartrate solution, to 50 ml with sodium acetate solution and measure the optical density after 10 minutes. Determine the reference value for propyl gallate by treating 10 ml portions of a solution containing 5 mg of propyl gallate per 100 ml of methanol in a similar manner.

The percentage of propyl gallate present is given by:

$$\frac{E_1 - E_2}{E_3 - E_4} \times \frac{1}{2p} \times \frac{110}{100}$$

where

E_1 = optical density of the water extract with ferrous tartrate solution,

E_2 = optical density of the water extract without ferrous tartrate solution,

E_3 = optical density of standard propyl gallate solution (500 μg) with ferrous tartrate solution,

E_4 = optical density of standard propyl gallate solution without ferrous tartrate solution, and

p = weight in g of ghee taken.

2) *Estimation of octyl and dodecyl gallate* — Pipette 20 ml of the water extract in a 150 ml separating funnel. Add 1 ml of ferrous tartrate solution and 40 ml of sodium acetate solution, and mix well. After 10 minutes add 20 ml of a mixture of equal parts of *iso*amyl alcohol and light petroleum, and shake vigorously for 2 minutes. Extract the lower layer, after separation, again for 2 minutes with another 20-ml portion of the mixture of equal parts of *iso*amyl alcohol and light petroleum. Run off the remaining water from the combined extracts as completely as possible, and transfer to a 50-ml flask. Add 3 ml of methanol to obtain a clear solution dilute to volume with *iso*amyl alcohol and mix well. Measure the optical density of the solution at 550 mμ relative to that of *iso*amyl alcohol, using an absorptiometer and 2-cm cell. Determine the optical density of the fat

extract alone by 20 ml of the methanol extract in the same way, omitting the ferrous tartrate solution. Determine the reference value for either octyl or dodecyl gallate by treating 10 ml portions of a solution containing 5 mg of the gallic acid ester in 100 ml of methanol in the same way. If it is not known which galiate is present, octyl and dodecyl gallate may be distinguished from each other by adding 1·5 ml of ferrous tartrate reagent solution to 5 ml of the methanol extract. After 5 minutes add 1 ml of a mixture of equal parts of *iso*amyl alcohol and light petroleum. Shake the mixture carefully (shaking too violently considerably delays the separation of the phases). If the upper layer becomes violet-blue in colour dodecyl gallate is indicated, since no colour is formed with octyl gallate. Note that, if the optical density of the methanol extract is more than one and a half times that of the reference solution, the procedure should be repeated with 2 ml instead of 1 ml of ferrous tartrate solution. The percentage of gallate present in the ghee is given by:

$$\frac{E_1 - E_2}{E_3 - E_4} \times \frac{1}{2p} \times \frac{V_1}{100} \times \frac{10}{V_2}$$

where

E_1 = optical density of the methanol extract with ferrous tartrate solution,

E_2 = optical density of the methanol extract without ferrous tartrate solution,

E_3 = optical density of standard gallate solution (500 µg) with ferrous tartrate solution,

E_4 = optical density of standard gallate solution without ferrous tartrate solution,

p = weight in g of ghee taken,

V_1 = final volume of the methanol extract (100 or 125 ml), and

V_2 = volume of the methanol extract taken for the determination.

NOTE — If other antioxidants, such as butylated hydroxyanisole or butylated hydroxytoluene, were also present in ghee, they pass into the methanol phase together with the gallates on being shaken with methanol. Both antioxidants, however, at the usual concentration of 0·02 percent, had no adverse effect on determinations of the gallic acid esters.

The influence of the synergists citric acid, ascorbic acid and phosphoric acid on the determination of the higher gallates was

completely identical with their effects on the determination of propyl gallate and will only be apparent if ghee is extracted directly with methanol. Normally these materials will be removed in the water extract with the propyl gallate.

20. DETECTION OF VEGETABLE FAT IN GHEE BY THE PHYTOSTERYL ACETATE TEST

20.0 The method is suitable for the detection of the presence of the most commonly used vegetable fats in ghee. The sensitivity depends upon the character of the vegetable fat used for admixture. The sterol content is determined gravimetrically after saponification of the fat and precipitation of the sterols by adding an alcoholic digitonine solution to the soap solution. The melting point of the sterol acetate is determined after acetylating the sterol digitonide by acetic anhydride. The crystal form of the sterols is microscopically examined after converting the sterol acetates into the steroids by saponification with an alcoholic potassium hydroxide solution.

20.1 Reagents

20.1.1 *Potassium Hydroxide (Analytical Grade) Solution* — dissolve 400 g of potassium hydroxide in 600 g of distilled water.

20.1.2 *Digitonine (Analytical Grade) Solution* — dissolve 10 g of digitonine in one litre of 96 percent ethanol (*v/v*).

20.1.3 *Ethanol* — redistilled, 95-96 percent (*v/v*) and 80 percent (*v/v*).

20.1.4 *Diethyl Ether*

20.1.5 *Acetic Anhydride*

20.2 Apparatus

20.2.1 *Conical Flask* — of 250 ml capacity with air-cooled condenser.

20.2.2 *Microfiltering Device*

20.2.3 *Melting-Point Apparatus*

20.2.4 *Melting-Point Tubes* — internal diameter 0·8 to 1·0 mm, length 50 mm.

20.2.5 *Microscope Slides and Cover Slips*

20.2.6 *Microscope* — linear magnification 200 ×.

20.2.7 *Thermometer* — reading up to 150°C with 0·1 deg graduations.

20.3 Procedure

20.3.1 *Determination of the Total Sterol Content* — Weigh accurately to the nearest 10 mg about 15 g of the ghee in a conical flask of 250 ml capacity. Add 10 ml of potassium hydroxide solution and 20 ml of 95 to 96 percent ethanol (*v/v*). Add 2 glass beads. Place the air-cooled condenser on the flask, heat on a boiling water-bath until the solution has become clear, and continue boiling for half an hour. Add 60 ml of water and then 180 ml of 96 percent ethanol (*v/v*), and raise the temperature to about 40°C.

20.3.1.1 Add 30 ml of the alcoholic digitonine solution (one percent), shake and allow to cool. Place the flask in a refrigerator at about 5°C for about 12 hours. Collect the precipitate of sterol digitonide by filtering through a filter paper (Whatman No. 1 or equivalent) in a Buchner funnel (diameter 8 cm). Wash out the precipitate with water at about 5°C until the filtrate stops foaming, then once with 25 to 50 ml of 96 percent ethanol (*v/v*) and at last once with 25 to 50 ml of diethyl ether. Dry the filter paper with precipitate on a watch-glass in a drying oven at 102° ± 2°C for about 10 to 15 minutes. Fold the filter into two, allowing the precipitate to come off as a pellicle, transfer the precipitate into a weighing bottle and weigh.

20.3.2 *Preparation of the Sterol Acetates and Determination of the Melting Point* — Transfer 100 ± 5 mg of the sterol digitonide to a test-tube, add 1 ml of acetic anhydride, and heat the tube in a glycerol-bath at 145°C until the precipitate has dissolved. Do not use direct heat, since spattering may occur. Continue heating for 2 minutes and allow to cool at about 80°C. Add 4 ml of 96 percent ethanol (*v/v*), mix, heat slightly to dissolve any steryl acetate which may tend to crystallize out. Filter the still warm solution through a small medium speed filter paper impregnated with ethanol, and collect the filtrate in another test-tube. Heat the filtrate in the latter test-tube and carefully bring to gentle boiling. While still boiling add carefully, drop by drop from a pipette 1 to 1·5 ml of water until the steryl acetate is just about to precipitate but still remains in solution. Avoid superheating. Add a few drops of 95 to 96 percent ethanol (*v/v*) to dissolve again any precipitated sterol acetate. Allow to cool in air for 2 hours, and finally in ice-water for half an hour. Filter the crystallized steryl acetates on a small disc of fast speed filter paper by suction in a glass microfiltering device and rinse the crystals with 1 ml of 80 percent ethanol (*v/v*). Redissolve the crystal cake by heating on a microburner in 1 ml of ethanol (96 percent) in a short heat-resistant glass test-tube (diameter 12 mm, length 35 mm). Allow to cool first in air for 15 minutes and then in ice-water for 5 minutes. Filter the crystallized sterol acetates as described above. Repeat redissolving, crystallization and filtration to obtain the third, occasionally the fourth or fifth recrystallization. Dry the crystal cake on the paper first at about 30°C in a drying oven and then at 102° ± 2°C in drying oven for 10 to 15 minutes.

Grind the crystal cake in a small agate mortar to make a finely divided powder and fill a melting-point tube to a height of about 3 mm. Determine the melting point in the melting-point apparatus raising the temperature very slowly in the last phase of the melting process at a rate of 0·5 degree per minute. Take the reading on the thermometer at the moment that the last crystal grain has just disappeared, at the melting point.

20.3.3 *Microscopic Examination of the Sterols* — Dissolve about 10 mg of the sterol acetate in a small test-tube in 1 ml of 96 percent ethanol (*v/v*) and add 1 or 2 drops of potassium hydroxide solution. Heat on a boiling water-bath until boiling begins and the steryl acetate has dissolved. Add 10 ml of distilled water, transfer the solution to a 125-ml separating funnel and shake with 25 ml of diethyl ether. After separation, drain and discard the aqueous layer. Wash the ether layer with three 5-ml portions of distilled water. Transfer the ether layer to a 50-ml beaker and evaporate to dryness. Dissolve the residue in 10 ml of 80 percent ethanol (*v/v*). Place a drop of the clear solution on a microscope cover slip, wait until crystallization starts on the periphery of the drop, then invert the cover slip and lay it on a microscope slide. Examine the crystals under the microscope at 200 × linear magnification. A diagram of the crystal shapes of sterol is given in Fig. 5.

20.4 Expression of Results

20.4.1 *Calculation*

$$\text{Total sterol content, percent} = \frac{0{\cdot}25 \times b}{a} \times 100$$

where

a = weight in g of ghee sample, and

b = weight in g of the sterol digitonide.

20.4.2 If the melting point of the steryl acetate is found to be 115°C, the ghee sample is considered to be free from vegetable fat. If the melting point of the sterol acetate is found to be higher than 117°C, the fat sample is considered to contain vegetable fat. If the melting point of the sterol acetate is found to be lower than 117°C and higher than 115°C, the fat sample is only considered to contain vegetable fat if the melting point is increased after replicate recrystallization.

20.4.3 If under the microscope the sterol crystals only show the form of a parallelogram with an obtuse angle of 100°, which is characteristic for cholesterol, the fat sample is considered to be free from vegetable fat.

If under the microscope the sterol crystals also show the elongated hexagonal form with an apical angle of 108°, which is characteristic for phytosterols, or if some of the sterol crystals have a re-entry angle (swallow's tail), which is characteristic for mixtures of cholesterol and phytosterols, the fat sample is considered to contain vegetable fat.

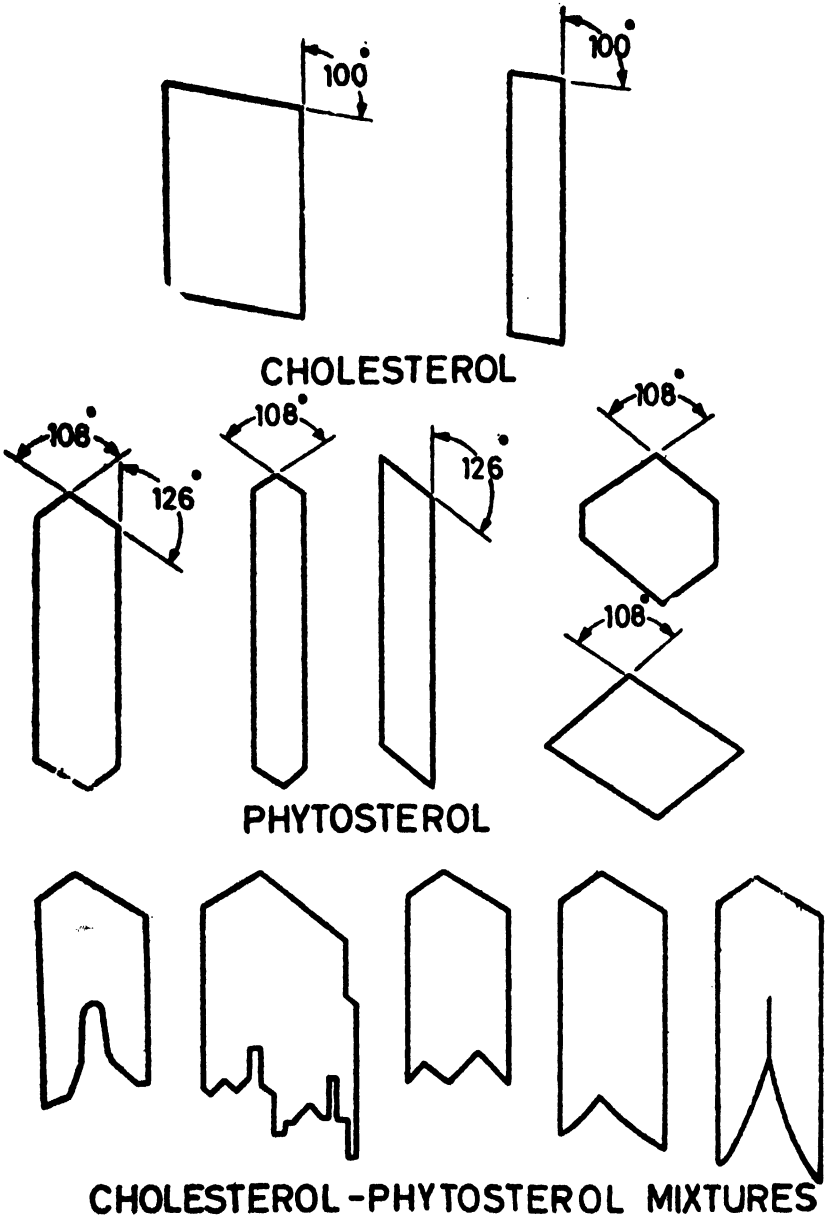


FIG. 5 CRYSTAL SHAPES OF STEROL

20.4.4 *Test Report*

20.4.4.1 The test report shall state the total sterol content expressed as a percent by weight, the melting point of the steryl acetate and the number of recrystallizations, and a description of the microscopic image of the sterol crystals.

21. DETERMINATION OF PRESENCE OF SESAME OIL (BAUDOUIN TEST)

21.0 The development of a permanent pink colour with furfural solution in the presence of hydrochloric acid indicates the presence of sesame oil.

21.1 Reagents

21.1.1 *Hydrochloric Acid* — fuming, sp gr 1·19.

21.1.2 *Furfural Solution* — 2 percent solution of furfural, distilled not earlier than 24 hours prior to the test, in rectified spirit (conforming to IS : 323-1959*).

21.2 **Procedure** — Take 5 ml of the melted ghee in a 25-ml measuring cylinder (or test-tube) provided with a glass stopper, and add 5 ml of hydrochloric acid and 0·4 ml of furfural solution. Insert the glass stopper and shake vigorously for two minutes. Allow the mixture to separate. The development of a pink or red colour in the acid layer indicates presence of sesame oil. Confirm by adding 5 ml of water and shaking again. If the colour in acid layer persists, sesame oil is present. If the colour disappears it is absent.

22. DETERMINATION OF THE PEROXIDE VALUE

22.0 The peroxide value is a measure of the oxidative rancidity in ghee and is expressed as millilitres of 0·002 N sodium thiosulphate per gram of sample, or as milliequivalents of peroxide oxygen per kilogram of sample. Two methods are recommended.

22.1 Iodometric Method

22.1.1 *Apparatus*

22.1.1.1 *Test-tubes* —150 × 25 mm. Before use, wash these thoroughly with soap solution, rinse with hot water and allow to stand in chromic acid mixture for a few hours. Then rinse thoroughly (the last time with distilled water) and dry in an oven before use.

22.1.1.2 *Rubber bung* — To fit the test-tube with a hole in the centre through which is inserted a small glass rod (of 3 to 4 mm diameter) flattened at one end and rounded off at the other.

*Specification for rectified spirit (*revised*).

22.1.1.3 *Water-bath*

22.1.1.4 *Conical flask* — 250 ml capacity.

22.1.2 *Reagents*

22.1.2.0 All reagents shall be of analytical grade.

22.1.2.1 *Solvent mixture* — a mixture of 2 volumes of glacial acetic acid and 1 volume of chloroform.

22.1.2.2 *Sodium thiosulphate* — 0·002 N solution, freshly prepared dilution from an accurately standardized 0·1 N solution.

22.1.2.3 *Potassium iodide* — freshly powdered.

22.1.2.4 *Potassium iodide* — 5 percent solution, freshly prepared.

22.1.2.5 *Starch indicator* — as in **14.3.4.**

22.1.2.6 *Carbon dioxide*

22.1.3 *Procedure* — The test should preferably be carried out in artificial light free from ultra-violet radiation. Weigh quickly but accurately a suitable quantity of the sample (the weight of the sample taken for the test should be such that the titration does not exceed 10 ml) into the test-tube and while still liquid add 1 g of powdered potassium iodide and 20 ml of the solvent mixture. Gently bubble carbon dioxide through the mixture of the ghee and solvent (for routine tests, this is unnecessary). Heat the contents of the tube to boiling within 30 seconds, preferably in a steam-bath, and allow them to boil vigorously for not more than 30 seconds. As the solvent vapours begin to escape from the hole in the bung, close the opening with the glass rod. Cool immediately under a tap and transfer into conical flask containing 20 ml of 5 percent aqueous solution of potassium iodide and wash out the test-tube twice with 25 to 30 ml of distilled water. Titrate the solution with the sodium thiosulphate solution using starch indicator. Do not add the starch until the end point is almost reached. Perform a blank test. This titration should not be more than 0·1 ml.

22.1.4 *Calculation*

22.1.4.1 Peroxide value $= 8\,000 \frac{A\,N}{M}$

where

A = volume of sodium thiosulphate solution required for the sample,

N = exact normality of the solution, and

M = mass in g of the sample taken.

22.1.4.2 Results may be expressed in millimolecules of oxygen per kg of fat. To obtain this, divide the peroxide value by 16.

22.1.4.3 The results expressed in milliequivalents of oxygen per kg of fat shall be obtained by dividing the peroxide value by 8.

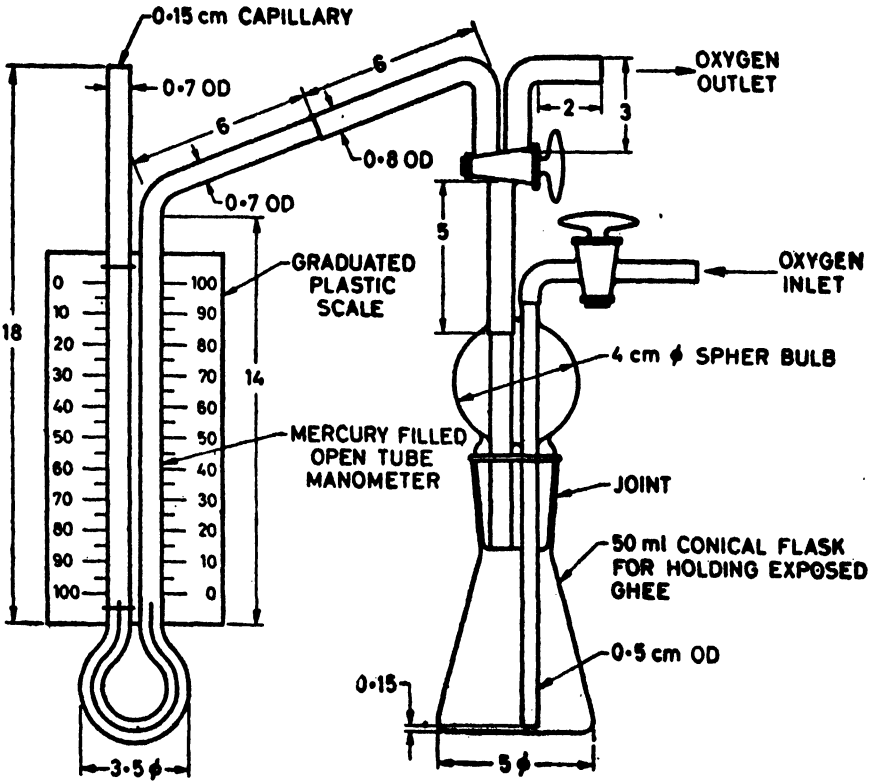
22.2 Oxygen Absorption Method

22.2.1 *Apparatus*

22.2.1.1 *Oxygen absorption apparatus* — as shown in Fig. 6.

22.2.1.2 *Oxygen gas*

22.2.1.3 *Oil-bath* — maintained at 79° ± 1°C.



All dimensions in centimetres.

FIG. 6 ALL-GLASS OXYGEN ABSORPTION APPARATUS

22.2.2 *Procedure* — Clean all glass parts with chromic acid. Rinse in distilled water and dry. Weigh accurately 5 g of ghee into conical flask and attach the manometer assembly. Open the inlet for oxygen and close connection to the manometer. Leave the outlet for oxygen open. Connect the inlet for the oxygen to the cylinder and regulate the flow of oxygen to bubble slowly through the melted ghee, continue fluxing with oxygen for

5 minutes. Close the inlet and disconnect the oxygen cylinder. Set the flask in the oil-bath at 79° ± 1°C. Open the flask to the manometer and periodically release the pressure inside the flask by opening the outlet for oxygen. Continue releasing the arms pressure inside the flask until equilibrium is reached between two arms of the manometer and the flask has attained the temperature of the oil-bath 15 to 20 minutes from the time of introduction of the flask in the oil-bath.

22.2.3 Note time when equilibrium is reached. Record reading of the manometer at the intervals of 2 hours initially in the fresh samples and one hour in case of old samples as well as the samples from stored butter and cream.

22.2.4 Note down the time when the manometer level in limbs connected to the flask starts its progressive rise, continue recording reading of level in manometer for another one hour. The number of hours elapsed after the equilibrium in the manometer was reached and the time when mercury level in manometer started progressive increase (up to 10 mm) corresponds to the induction period of the sample.

22.2.5 Value for induction period of 20 hours and over appears to correspond to a marketable life of 6 months. Samples having an induction period below 6 hours are found to be unmarketable.

22.3 Interpretation of Results — Whilst either the iodometric peroxide value or the induction period as determined by the oxygen absorption method could be used to measure the keeping quality (shelf life) of ghee a combination of both gives the most reliable results:

Peroxide Value ml of 0·002 N Sodium Thiosulphate Solution/g	*Interpretation of Quality*	*Induction Period at 79°C in Hours*	*Peroxide Value of Exposed Sample at the End of Induction Period*
Below 1·5	Very good	Above 20	Below 18·0
1·6 to 2·0	Good	16-20	Below 21·0
2·1 to 2·5	Fair	11-15	Below 24·0
2·6 to 3·5	Poor	6-10	Below 27·0
3·6 to 4·0	Not acceptable	Below 6	Above 30·0

23. DETERMINATION OF IRON CONTENT

23.0 General — This method is considered satisfactory for the determination of iron occurring normally in ghee. Acid extraction suffices for this case and has been found preferable to the ashing technique. Where, however, the presence of iron from extraneous sources in less soluble inorganic forms, for example, ferrosilicate, is suspected, total iron should be determined by ashing and alkali fusion.

23.1 Principle of Method

23.1.1 Colorimetry of the pink colour formed by reaction of Fe^{+++} with thioglycollic acid.

23.2 Reagents

23.2.0 The reagents used shall be of analytical reagent quality and free from iron. Distilled water, re-distilled from all-glass apparatus, shall be used throughout.

23.2.1 *Hydrochloric Acid* — Sp gr 1·18.

23.2.2 *Ammonia Solution* — Sp gr 0·88.

23.2.3 *Thioglycollic Acid*

23.2.4 *Bromine Water* — saturated.

23.2.5 *Standard Iron Solution* — Dissolve 8·635 g of ammonium ferric sulphate, $NH_4Fe(SO_4)_2.12H_2O$ in distilled water containing 5 ml of dilute (1 : 3) sulphuric acid and dilute to 1 000 ml. Before use, dilute this stock solution one hundred times by successive dilutions. One millilitre of the resulting solution is equivalent to 10 microgrammes Fe.

23.3 Apparatus

23.3.1 *Flask* — of 250 ml capacity, with interchangeable conical ground glass joints.

23.3.2 *Spectrophotometer or Photoelectric Absorptiometer* — With a blue-green filter having a maximum transmission at approximately 480 nm; all-glass cells should be used and should be of such size (1-cm cells are usually satisfactory) that the optical density of the solution under test lies between 0·1 and 0·8.

or

23.3.3 *Nessler Cylinders* — of 50-ml capacity.

23.4 Procedure

23.4.1 Weigh 25 g of the sample into the flask. Add 15 ml of water and 20 ml of hydrochloric acid. Use a few glass beads to regulate the boiling. Reflux for one hour. Transfer to a separating funnel, allow to settle and run off the aqueous layer through a double, acid-washed, medium texture filter paper into a 300-ml beaker (a Whatman No. 40 paper or equivalent, washed with hydrochloric acid of the same concentration as that used for the extraction immediately before use, is suitable). Wash the sample again in the separating funnel with two 50-ml portions of hot water, using these to rinse the flask and pouring them through the filter after washing the sample. Evaporate the combined aqueous and acid extracts to about 5 ml and add 5 ml of bromine water.

23.4.2 Boil gently until the excess bromine is driven off. When cool, transfer to a Nessler cylinder. Add 2 to 3 drops of thioglycollic acid, make just alkaline to litmus by the dropwise addition of ammonia solution and dilute to 50 ml with water. Read the colour in the absorptiometer or in the spectrophotometer.

23.5 Calculation and Expression of Results

23.5.1 Determine the iron content of the solution from a standard curve prepared as follows:

Measure into five separate 50-ml graduated flasks by means of a burette, nil, 5, 10, 15 and 25 ml of the standard iron solution. Add 5 ml of water, 3 ml of the hydrochloric acid, 2 or 3 drops of thioglycollic acid and ammonia solution dropwise until the solutions are just alkaline to litmus paper. Cool, dilute to the mark with distilled water and mix well. Determine the colours of the solutions in the appropriate instrument, using in the reference cell the control solution containing no iron. Plot a curve correlating transmission or optical density against micrograms of iron.

23.5.2 Alternatively, the iron content may be determined by matching with standards in Nessler cylinders, as follows:

Place in a 50-ml Nessler cylinder, 35 ml of water, 3 ml of hydrochloric acid and 2 to 3 drops of thioglycollic acid and add ammonia solution until the solution is just alkaline to litmus paper. Cool, dilute to 50 ml with water and add, from a burette, with constant stirring, standard iron solution until the colour of the solution matches that of the test solution. Note the volume of standard iron solution used. Repeat the preparation of the matching solution, but place the volume of standard iron solution previously determined in the Nessler tube before adding the reagents. If the standard colour is now different from that of the test solution, prepare other standards containing appropriately more or less iron until a perfect match is obtained. The best visual matches are obtained when the solution in the Nessler cylinder contains 1 to 2 ml of standard iron solution.

23.5.3 Express the results as mg iron/kg of the sample.

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